Dissection of a functional interaction between the DNA translocase, FtsK, and the XerD recombinase

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Summary
Successful bacterial circular chromosome segregation requires that any dimeric chromosomes, which arise by crossing over during homologous recombination, are converted to monomers. Resolution of dimers to monomers requires the action of the XerCD site-specific recombinase at dif in the chromosome replication terminus region. This reaction requires the DNA translocase, FtsK, which activates dimer resolution by catalysing an ATP hydrolysis-dependent switch in the catalytic state of the nucleoprotein recombination complex. We show that a 62-amino-acid fragment of FtsK C-terminus activates directly with the XerD C-terminus in order to stimulate the cleavage by XerD of BSN, a dif-DNA suicide substrate containing a nick in the ‘bottom’ strand. The resulting recombinase–DNA covalent complex can undergo strand exchange with intact duplex dif in the absence of ATP. FtsK-mediated stimulation of BSN cleavage by XerD requires synaptase complex formation. Mutational impairment of the XerD–FtsK C-interaction leads to reduction in the in vitro stimulation of BSN cleavage by XerD and a concomitant deficiency in the resolution of chromosomal dimers at dif in vivo, although other XerD functions are not affected.

Introduction
After termination of bacterial chromosome replication, the two sister chromosomes need to be physically separated before segregation can be completed. Two potential impediments to such separation are catenation of the two monomeric sister chromosomes and circular chromosome dimer formation by crossing over during homologous recombination (reviewed in Espeli and Marians, 2004; Barre and Sherratt, 2005). Topoisomerase action is required for decatenation, while XerCD site-specific recombination at the recombination site dif, located in the ter region converts bacterial chromosome dimers to monomers. FtsK, a 1329-amino-acid integral membrane protein that localizes to the FtsZ ring, functions directly in dimer resolution and facilitates decatenation by topoisomerase IV (Espeli et al., 2003). In addition, FtsK may contribute directly to decatenation during XerCD recombination at dif (Ip et al., 2003). The 179-amino-acid N-terminal membrane domain of FtsK is required for cytokinesis, while the ~500-amino-acid C-terminal domain (FtsK C) is a DNA translocase that functions in chromosome segregation and dimer resolution (Draper et al., 1998; Yu et al., 1998; Aussel et al., 2002). Therefore, FtsK links chromosome segregation with cell division via its C- and N-terminal domains. The DNA sequence-directed translocation activity of FtsK C promotes synopsis of dif sites in the vicinity of the septum before recombination activation (Péral et al., 2000; Bigot et al., 2004; Barre and Sherratt, 2005; Pease et al., 2005). FtsK C action may also facilitate chromosome segregation by organizing newly replicated ter regions at midcell (Lesterlin et al., 2004).

It has been proposed previously that FtsK C activates XerCD recombination at dif by catalysing an ATP-dependent switch in the conformation of XerCD–dif nucleoprotein complex, thereby allowing XerD to initiate recombination (Aussel et al., 2002). Here we dissect the activation process by showing that a biochemically active form of FtsK C, FtsK C (Aussel et al., 2002), can stimulate cleavage by XerD of a dif-DNA suicide substrate, BSN, containing a nick in the ‘bottom’ strand. This occurs only in synaptic complexes containing two BSN DNA fragments. Furthermore, we show that interaction between a 62-amino-acid non-motor subdomain of FtsK C and the C-terminus of XerD is required for stimulation of BSN cleavage. This stimulation can also lead to intermolecular recombination between BSN and intact linear dif duplex in the absence of ATP.
Results and discussion

FtsK<sub>50C</sub> stimulates XerD-mediated cleavage of BSN DNA

Previous work has demonstrated that FtsK<sub>50C</sub>-dependent activation of a complete Xer recombination reaction at dif requires ATP hydrolysis by FtsK<sub>50C</sub> and a DNA extension adjacent to the XerD binding site on at least one of the two participating dif sites (Aussel et al., 2002; Massey et al., 2004). This reaction requires a specific interaction between FtsK<sub>50C</sub> and the XerCD recombinase that leads to an ATP hydrolysis-dependent switch in the catalytic state of the recombination complex, so that recombination is initiated by XerD to give a Holliday junction (HJ) intermediate that is resolved to complete recombinant product by XerC (Aussel et al., 2002; Yates et al., 2003).

In order to dissect the molecular basis of this activation mechanism, we designed experiments to test whether FtsK<sub>50C</sub>, or parts of it, could activate the initial chemical step of the recombination reaction, cleavage of DNA by XerCD to form a recombinase–DNA covalent complex. Short DNA substrates containing the 28 bp dif site with a nick in either of the two strands at the centre of the dif site can be used to monitor recombinase-mediated DNA cleavage, as after cleavage a trinucleotide can diffuse away, or the DNA can dissociate into two fragments, thereby capturing the covalent complex between the recombinase and dif (Fig. 1A; Nunes-Düby et al., 1987; Blakely et al., 2000).

Whereas in vitro recombination between intact dif sites requires FtsK<sub>50C</sub>, ATP hydrolysis and a DNA extension on the XerD binding site of dif (Aussel et al., 2002; Massey et al., 2004), XerD-mediated cleavage of BSN, a 54 bp

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**Fig. 1.** FtsK<sub>50C</sub> stimulates XerD-mediated cleavage of BSN.
A. Schematic of XerCD-mediated cleavages of nicked suicide substrates, BSN and TSN. XerC (circle) binds to the left half-site of dif while XerD (square) binds to the right half-site. A 6 bp central region separates the two binding sites. The 28 bps dif site is flanked by 17 bp or 13 bp DNA segments. XerC and XerD cleave the top (grey) and bottom (black) strand of dif respectively. The position of the 5′ radiolabel is indicated by a star. The BSNe substrate carries a 205 bp extension adjacent to XerD binding site (not shown). Cleavage by either recombinase gives a diagnostic labelled DNA fragment with covalently attached recombinase.

B. The 60 min 37°C reactions of the indicated reagents were analysed on a 0.1% SDS-6% PAGE. The levels of FtsK<sub>50C</sub>-mediated stimulation of DNA cleavage by XerD and XerC are shown, as are the ratios of cleavage by XerD as compared with XerC. The levels of FtsK<sub>50C</sub>-mediated stimulation of DNA cleavage by XerD varied between 2.5- and 32-fold after 60 min reactions in different experiments. In part, this results from day-to-day variations in FtsK<sub>50C</sub>-specific activity. Within experiments using a given set of protein dilutions, cleavage activities can be reliably compared.

C. Time-course of XerD-mediated cleavage of BSN substrate in the presence and absence of FtsK<sub>50C</sub>. The percentage of substrate DNA converted to BSN-XerD with respect to time is plotted. The level of stimulation by FtsK<sub>50C</sub> as judged by initial rates is >10-fold in this experiment.

D. XerCD cleavage reactions with TSN substrate.
dif substrate with a nick in the bottom strand, was stimulated by FtsK_{50C}, in a reaction that was independent of ATP (Fig. 1B and C). Consistent with this, Walker A and Walker B mutants of FtsK_{50C}, which should be unable to bind ATP, or hydrolyse ATP, respectively, were also able to stimulate cleavage by XerD (data not shown). Addition of a 200 bp extension to the XerD binding side of BSN generated BSNe, whose cleavage by XerD was stimulated by FtsK_{50C} to a comparable extent as BSN. We conclude that activation of BSN cleavage by XerD does not require the loading of FtsK_{50C} onto duplex DNA, as the 13 bp flank adjacent to the XerD binding site of BSN is insufficient to facilitate FtsK_{50C} loading onto DNA (Massey et al., 2004).

There was no stimulation of XerC-mediated cleavage by FtsK_{50C} on these substrates (Fig. 1B), consistent with our demonstration that FtsK_{50C} activates XerD in a complete XerCD dif intermolecular recombination reaction (Aussel et al., 2002). Furthermore, FtsK_{50C} did not stimulate XerD-mediated cleavage of TSN, a substrate with the nick in the top stand of the dif central region that undergoes cleavage by XerC preferentially (Fig. 1D).

These results suggest that a configuration in which XerD is potentially active within synaptic complexes can more readily be adopted with BSN than with duplex dif and that the formation of such synaptic complexes with BSN is stimulated by FtsK_{50C} without the need for ATP hydrolysis.

A 62-amino-acid fragment of FtsK_{C} is sufficient to stimulate cleavage of BSN by XerD

In vivo experiments designed to map which region of FtsK_{C} is responsible for a productive interaction with XerCD–dif identified a 142-amino-acid region of FtsK_{C} that contains sequences responsible for species specificity in this interaction. This region of Escherichia coli (Ec) FtsK_{C} is responsible for interacting specifically with the Ec Xer recombination machinery while the equivalent domain derived from Haemophilus influenzae (Hi) interacts specifically with the Hi Xer recombination machinery (Yates et al., 2003). The 142-amino-acid region contains an 81-amino-acid C-terminal segment that is relatively non-conserved in sequence between Ec and Hi (residues 1249–1329). We define this as the \( \gamma \) subdomain of FtsK_{C} (Fig. 2A). The C-terminal 126 residues of Ec FtsK_{C}, containing \( \gamma \), were fused to maltose-binding protein (MBP\( \gamma \); FtsK residues 1203–1267), MBP\( \gamma 1 \) (residues 1203–1267), and MBP\( \gamma 2 \), (residues 1268–1329).

Purified MBP\( \gamma \) and MBP\( \gamma 2 \) each stimulated DNA cleavage of BSN by XerD, whereas MBP\( \gamma 1 \) showed no activity (Fig. 2B). Activation was independent of ATP hydrolysis, as expected for proteins lacking the ATP-dependent motor \( \beta \) domain of FtsK_{C}. We conclude that determinants for interaction with XerCD reside in the C-terminal 62 amino acids of Ec FtsK_{C}, and that these are sufficient to stimulate BSN cleavage by XerD.

**The FtsK_{C} \( \gamma \) subdomain promotes intermolecular XerCD-dependent strand exchange between BSN and intact duplex dif**

As a consequence of the ability of \( \gamma \) to stimulate cleavage...
of BSN by XerD, we reasoned that FtsK50C and γ might be able to stimulate strand exchange between BSN and an intact \textit{dif} duplex, without the need for ATP. In order to test this, recombinase-mediated cleavage and strand exchange in reactions containing radiolabelled BSN, and an unlabelled intact DNA fragment containing \textit{dif} flanked by 197 bp and 200 bp DNA segments (197\textit{dif}200) were assayed. Incubation of the two DNA substrates with XerCD and FtsK50C in the absence of ATP generated, in addition to the expected recombinase–BSN covalent complexes, a spectrum of novel radiolabelled DNA species, most of which were dependent on FtsK50C (Fig. 3A and B).

The same recombinant profile was obtained with FtsK50C and MBPγ (Fig. 3A), confirming that this intermolecular recombination is independent of the translocation activity of FtsK50C. A comparison of product profiles before and after proteinase K treatment showed which products had recombinase covalently bound, while experiments utilizing maltose-binding fusion derivatives of the recombinases determined which covalent complexes with DNA had XerC or XerD bound (data not shown).

A Holliday junction with XerD covalently bound (HJ-D) formed as quickly as BSN-D when FtsK50C was present (Fig. 3B, right). Therefore, intermolecular complexes can form and react efficiently under the reaction conditions used. Assuming that the FtsK50C-dependent products arise from reactions initiated by XerD, HJ-D, which has a 3 nt gap, must have arisen from two XerD-mediated cleavages, one of which is accompanied by strand exchange (Fig. 3C). Nicked HJs, which are present in low amount only at later times, could result from two completed strand exchanges by XerD; this is expected to occur inefficiently as it would require that the 3 nt oligonucleotide be retained in the recombining complex. Both HJ-D and HJ can be processed by a pair of XerC-mediated strand exchanges, generating linear recombinant products, LP-D and LP

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**Fig. 3.** The FtsK-γ subdomain can stimulate intermolecular recombination between BSN and intact \textit{dif}-DNA substrates.

A. The 60 min 37°C reactions containing radiolabelled BSN and 197\textit{dif}200, an unlabelled intact linear DNA duplex, and the indicated reagents were analysed by 0.1% SDS-4% PAGE. BSN-C, covalent complex of BSN fragment and XerC; BSN-D, covalent product of BSN and XerD; LP, nicked or gapped recombinant product of BSN and 197\textit{dif}200; LP-D, XerD covalent complex of gapped LP; HJ-C, covalent complex of XerC with a HJ that has lost a DNA arm; HJ-D, covalent complex of XerD and a gapped HJ (see C).

B. Time-course of reaction of radiolabelled BSN, 197\textit{dif}200 DNA, and XerCD in the absence (left) and presence of FtsK50C (right). DNA species as labelled in (A).

C. The proposed sequence of steps leading to generation of the observed radiolabelled products initiated by XerD. Completion of a pair of strand exchanges by XerD requires that the 3 nt fragment (represented by three dots) released by BSN cleavage by XerD does not diffuse out of the synaptic complex so that its 3’ end can be used to attack the phosphotyrosyl bond on 197\textit{dif}200. A gapped HJ can arise by hydrolysis of the phosphotyrosine in HJ-D. The nick in BSN and subsequent products is shown by a small arrow.
respectively. We imagine that it is easier for XerC strand exchanges to occur on HJ than on HJ-D. Formally, LP-D could also arise from XerD-mediated cleavage of linear nicked recombinant product (LP), although this is unlikely because LP-D accumulates before LP. Gapped LP or HJ could also arise from LP-D or HJ-D by phosphotyrosine hydrolysis.

Although the data on the relative amounts of reactions products and the kinetics of their production and turnover support the hypothesis that all of the observed FtsK_{C'}-dependent products arise from reactions that were initiated by XerD, we cannot yet formally prove that this is the case. Nevertheless, we note that if strand exchange by XerC precedes XerD in reactions with synapsed duplexes, it would generate labelled intermediates [nicked HJ (HJ) and XerC covalently linked to a three-armed junction (HJ-C)]. Consistent with this, HJ and HJ-C are the two FtsK_{C'}-independent products resulting from strand exchange between BSN and 197{diff}200 (Fig. 3B, left). We expect these reactions to be initiated by XerC. Although these initial products might be acted on by XerD to produce BSN-D, HJ-D, LP-D and LP in the presence of FtsK_{C'}, we believe this unlikely because the reaction kinetics of intermolecular product formation show that the products of XerD-mediated cleavage and strand exchange appear before any that could be produced by XerC (Fig. 3B, right). Furthermore, the kinetics of HJ-C and HJ appearance and turnover are not readily compatible with them being processed by XerD. Indeed, {diff} HJ intermediates made by XerC do not show significant resolution by XerD under any conditions tested and we have never been able to show stimulation of XerD activity by FtsK_{C'} on HJ intermediates (Arciszewska et al., 2000; Massey et al., 2004).

In contrast, intact or nicked {diff} HJs are excellent substrates for resolution by XerC (not shown).

These results on FtsK_{C'}-dependent intermolecular recombination support the view that the stimulation of XerD-mediated cleavage of BSN by FtsK_{C'} represents a reaction that is functionally relevant. Also consistent with this conclusion is the observation that FtsK_{C'}-dependent stimulation of BSN cleavage requires the presence of both XerC and XerD (data not shown).

**FtsK_{C'}-mediated stimulation of BSN cleavage by XerD occurs in synaptic complexes**

The results above, which show that intermolecular complexes containing two duplexes form and react quickly, are consistent with the idea that the FtsK_{C'}-dependent stimulation of BSN cleavage by XerD requires synaptic complex formation. In order to test this hypothesis, we first determined whether FtsK_{C'}-independent cleavage of BSN by XerC and XerD is synopsis-dependent, by assaying cleavage reaction efficiency on TSN and BSN as a function of DNA concentration. In all of the experiments, a constant level of radiolabelled BSN or TSN was mixed with unlabelled BSN or TSN in varying amounts before reaction with the indicated proteins. The 3 min, 15 min and 60 min reactions were analysed (Fig. 4 and not shown). The concentration dependence of the FtsK_{C'}-stimulated cleavage of BSN by XerD was essentially identical to BSN-D cleavage alone (the difference between the two BSN-D curves). FtsK_{C'} had no effect on the BSN reactions or on cleavage of BSN by XerC (not shown).
results were unambiguous with substrate TSN; cleavage by XerC is synapsis-dependent, the level of cleavage increasing as DNA concentration increases from 5 to 45 nM. In contrast, cleavage by XerD on this substrate does not increase as intermolecular reaction between two duplexes increases and therefore is synapsis-independent. The complementary result was obtained with BSN; cleavage by XerD is synapsis-dependent, while cleavage by XerC is synapsis-independent (not shown). Consistent with the synapsis-independent cleavage of BSN by XerC, we observed that high concentrations of XerC alone can cleave BSN; we would not expect that XerC alone can mediate synaptic complex formation. We note that in Cre–loxP recombination, the $K_0$ for synapsis is ~10 nM; our results are consistent with the $K_0$ for XerCD–dlf synthesis being of a similar order. If this is the case, at the high DNA and protein concentrations we use, much of the radiolabelled DNA will be in synaptic complexes (Ghosh et al., 2005).

Next, we tested whether the FtsK50C-stimulated cleavage of BSN by XerD is synapsis-dependent. Again the result was unequivocal; the stimulated cleavage like all catalysis by XerD on BSN is synapsis-dependent, with the concentration dependence being identical to that of catalysis by XerD on BSN in the absence of FtsK50C. This result provides a possible explanation of why FtsK50C fails to stimulate cleavage by XerD on TSN. The stimulation by FtsK50C requires formation of synaptic complexes that are in the XerD-active configuration; TSN cleavage by XerD is synapsis-independent. Also consistent with the synapsis-dependent cleavage of BSN by XerD is the observation that XerD alone can cleave BSN (not shown). This cleavage is not stimulated by FtsK50C, thereby reinforcing the view that FtsK50C-stimulated cleavage by XerD requires synapsis complex formation, as XerD alone is unlikely to mediate synapsis between two BSN fragments. Finally, FtsK50C failed to stimulate cleavage of BSN by XerD in the presence of XerC[Y275F], which is mutated for the catalytic tyrosine (not shown); the equivalent mutants of Cre are synapsis-defective (G.D. Van Duyne, pers. comm.).

The demonstration that cleavage of BSN by XerD, in the presence of XerC, is synapsis-dependent reinforces the view that all FtsK50C-mediated effects on XerCD recombination are mediated by promoting the formation of heterotetrameric synaptic complexes that have a conformation appropriate for catalysis by XerD (later). Furthermore, our observation with TSN and BSN that catalysis by one recombinase is synapsis-dependent and the other independent (with a switch in which recombinase requires synapsis as one goes from TSN to BSN) is reminiscent of the demonstration that in Cre–loxP recombination, ‘bottom strand’ cleavage in vitro requires synapsis, while ‘top strand’ cleavage is synapsis-independent, although in these latter experiments duplexes with a bridging phosphorothioate were used rather than nicked duplexes (Ghosh et al., 2005). Finally, our results show the impact that a strand-specific nick has on which productive synaptic complex can form and react. Similarly, the $\text{loxP}^\gamma$-Cre experiments demonstrate how specific synaptic complex formation directs a preferred order of strand exchange.

The $\gamma$ subdomain of FtsK$_C$ interacts with XerD

In order to determine which components of the XerCD–dlf recombination machinery are interacting with FtsK$_{50C}$. His-tagged derivatives of each of the recombinases were immobilized on cobalt-agarose affinity resin and tested for their ability to interact with FtsK$_{50C}$ and its derivatives. Initial experiments showed that His-tagged variants of either XerC or XerD retained their non-His-tagged recombinase partner on the affinity resin, with both recombinases being co-eluted by 300 mM imidazole, thereby demonstrating that a XerC–XerD interaction can be assayed in the absence of DNA (Fig. 5A).

Im mobilized HisXerD also specifically retained FtsK$_{50C}$, with both proteins co-eluting with imidazole (Fig. 5B, II). In contrast, pre-bound HisXerC did not retain FtsK$_{50C}$ (Fig. 5B, I), although pre-bound HisXerC retained FtsK$_{50C}$ in the presence of XerD. In this case, all three proteins co-eluted with imidazole (not shown). Additionally, XerD, but not XerC, was retained efficiently on the cobalt agarose resin pre-loaded by HisFtsK$_{50C}$ (Fig. 5B, III, IV). The small amount of XerC that co-elutes with FtsK$_{50C}$ could reflect a weak interaction between FtsK$_{50C}$ and XerC. We conclude that FtsK$_{50C}$ interacts strongly with XerD, irrespective of the presence of XerC. These interactions are ATP-independent.

MBP$_\gamma$ and MBP$_\beta^2$ behaved like FtsK$_{50C}$. They were retained by immobilized HisXerC–XerD, and co-eluted with XerCD in the presence of imidazole (Fig. 5C). This retention was not dependent on XerC when HisXerD was immobilized (not shown). MBP$_\gamma$ was not retained by immobilized HisXerD, thereby confirming that the $\gamma$ interaction with XerD is mediated by $\gamma^2$. FtsK$_{50C}$ bound to immobilized HisXerC–XerD could also be eluted by MBP$_\gamma$, confirming the specificity of the interaction (Fig. 5D). Confirmatory experiments using FLAG- or intein-tagged derivative of FtsK$_{50C}$ also showed specific binding of XerD, but not XerC (data not shown). We conclude that the 62-amino-acid $\gamma^2$ segment of FtsK$_C$ contains the determinants for the ATP-independent interaction with XerD.

The extreme C-terminus of XerD interacts with the $\gamma$ subdomain of FtsK$_C$

XerC and XerD belong to the tyrosine recombinase family,
which have relatively little amino acid sequence homology, despite being structurally highly related and sharing a common catalytic mechanism (Esposito and Scocca, 1997; Sherratt and Wigley, 1998). We have taken advantage of the 67% sequence divergence between XerC and XerD to construct chimeric recombinases that are part XerC and part XerD (Ferreira et al., 2003). These chimeras were exploited in order to identify which part of XerD interacts with FtsK50C by testing their ability to interact with immobilized HisFtsK50C (Fig. 6A). The results were unambiguous; the specificity of interaction with HisFtsK50C resided in the extreme C-terminal region of XerD (residues 282–298). Further analysis using XerD variants carrying deletions in the C-terminal region revealed that XerD deleted for its C-terminal five amino acids was proficient in interaction with FtsK50C (Fig. 6B). In contrast, a deletion of the

Fig. 5. The FtsKγ subdomain interacts directly with XerD.
A. XerCD interact in the absence of dif-DNA. Analysis by SDS-PAGE of recombinase interactions. 1. The indicated His-tagged recombinase is applied to cobalt agarose resin. L, protein loaded; F, flow through fraction; W, wash fraction. 2. Then the second indicated recombinase is applied to the washed column indicated in 1. E, proteins eluted by 300 mM imidazole; other abbreviations as in 1. Untagged XerCD did not bind to the columns under these binding conditions (controls).
B. FtsK50C interacts with XerD. The sequential loading of His-tagged recombinase or FtsK50C to cobalt agarose resin, column washing, and then application of a second non-His-tagged protein, followed by 300 mM imidazole elution was as in (A). I–IV show the indicated protein combinations. C. MBPγ2 interacts with XerD. Sequential loading, washing and elution of the indicated proteins were as in (A). The elutions only are shown. D. MBPγ specifically competes with FtsK50C. Sequential loadings and elutions as indicated.
Whether these residues are sufficient for the interaction of XerD with FtsK is located between amino acids 282 and 292. XerD carrying determinants necessary for interaction with FtsK were tested for their ability to bind to immobilized HisFtsK_{50C}. Per cent protein bound was calculated from percentage E/L. The indicated XerCD chimeras were tested for their ability to interact with immobilized HisFtsK_{50C} by using the sequential loading and elution protocol described in Fig. 5. Abbreviations as in Fig. 5A.

10 or 15 C-terminal residues gave a protein that no longer showed an interaction with FtsK_{50C}, despite being able to bind to dif-DNA (Fig. 6B; data not shown; Spiers and Sherratt, 1997). We therefore conclude that the region of XerD carrying determinants necessary for interaction with FtsK_{50C} is located between amino acids 282 and 292. Whether these residues are sufficient for the interaction is not known.

As Ec XerD interacts in vivo with Ec FtsK, but not with Hi FtsK_{50C} (Yates et al., 2003), we expected to find differences between Ec and Hi XerD in this C-terminal region. Five such differences identify amino acids that are unique to XerD, yet are absent in XerC (Fig. 7A). Intriguingly, this region of γ-proteobacterial XerD is relatively highly diverged as compared with the comparable region in XerC. We constructed Ec XerD variants carrying amino acids present in Hi XerD at four of the five positions (Fig. 7A). We assessed double and quadruple substitution variants for their ability to interact with immobilized HisFtsK_{50C} and for their ability to be stimulated by FtsK_{50C} in a dif-BSN cleavage assay. The results of both assays show that determinants for XerD interaction with FtsK_{50C} are located within these four residues (Fig. 7B). Variants XerD[KR] and XerD[ER], each carrying two substitutions, showed only slightly reduced activity, while the variant having the quadruple substitution, XerD[KR..ER], had an activity similar to Hi XerD, consistent with the species specificity residing in this region.

We reasoned that if these four amino acids are specifically involved in the interactions with FtsK_{50C}, their substitution should not affect the ability of Ec XerD to mediate recombinational DNA substrates that do not require activation by Ec FtsK_{50C}, for example, the plasmid recombination site, psi (Colloms et al., 1996). We therefore compared the proficiency of XerD and the XerD variants in supporting in vivo recombination between directly repeated plasmid-borne dif or psi recombination sites flanking a Km^{R} cassette (Fig. 7C). All of the XerD variants completely resolved the psi reporter plasmid. In contrast, XerD[KR..ER], containing the quadruple substitution, showed no detectable dif resolution (note that resolution at dif is generally less efficient than at psi with wild-type XerD). Transformation of the plasmid DNA into an Xer− strain allowed a quantitative measure of resolution at dif by scoring the ratio of Km^{R}/Sp^{R}/Km^{R} Sp^{R} transformants. Whereas 14% of dif reporter plasmids had resolved in a wild-type XerD^{+} strain, 2% had resolved with the strain carrying the XerD quadruple substitution (Fig. 7C).

Escherichia coli strains lacking chromosomal dif, functional xerCD or ftsK_{50C} genes, are defective in chromosome segregation, largely as a consequence of failing to resolve chromosome dimers (Recchia et al., 1999; Barre and Sherratt, 2005). We therefore reasoned that the presence of the Ec XerD[KR..ER] variant, which is unable to interact with Ec FtsK_{50C}, should lead to a defect in dimer resolution and consequent chromosome segregation, despite its ability to recombine plasmid psi sites. To test this we used a co-culture competition assay, which provides a sensitive measure of the efficiency of chromosome dimer resolution (Fig. 7D; Péral et al., 2000; Bigot et al., 2004). Two differentially marked strains (Tp^{R} or Km^{R}), lacking a functional copy of their chromosomal xerD gene, carried related plasmids that expressed either wild-type XerD or an XerD variant. After mixing the cultures in a 1:1 ratio they were grown to stationary phase, diluted 1000-fold, grown back to stationary phase and so on until 40 generations had been reached in medium that selects for main-
Fig. 7. XerD variants carrying substitutions at R288, Q289, Q292 and Q293 lack the ability to interact with FtsK<sub>Ec</sub>-<br>of cleavage. <br>A. Amino acid sequence alignment of C-terminal regions of Ec XerD, Hi XerD and Ec XerC. The amino acid residue 282–293 region of Ec XerD (horizontal line) has been shown by deletion analysis (Fig. 6) to be necessary for the interaction with FtsK<sub>Ec</sub>. Differences in amino acid sequence between Ec XerD and Hi XerD within this region are indicated (stars), as are the four residues targeted for mutagenesis (bold). The active site tyrosine (residue 279) is also indicated (ovoid). B. Interactions between the XerD variants and FtsK<sub>Ec</sub>. The indicated XerD variants were assayed for their ability to bind to immobilized HisFtsK<sub>Ec</sub> (open bars) and to stimulate cleavage of BSN in the presence of FtsK<sub>Ec</sub> (grey bars). The activity of the variants is expressed as a percentage of the activity of wild-type Ec XerD. C. In vivo analysis of XerCD recombination at plasmid-borne dif in the presence of XerD or its variants. The Sp<sup>R</sup> reporter plasmids carried either dif-Km<sup>R</sup>-dif (d) or psi-Km<sup>R</sup>-psi (p) cassettes in which two dif or two psi sites were directly oriented. Plasmid DNA preparations obtained from stationary-phase cultures of strains carrying reporter plasmids and vectors expressing XerD or XerD variants were analysed by agarose electrophoresis. The efficiencies of dif and psi plasmid resolution in the presence of XerD and XerD[KR..ER] were quantified by scoring for the loss of Km<sup>R</sup> marker upon transformation of the DNA samples shown into a Xer<sup>–</sup> strain. Five hundred transfor-<br>of chromosomal antibiotic resistance markers were exchanged led to <br>the same results (not shown).<br>D. Efficiency of chromosome dimer resolution by FtsK<sub>Ec</sub>-dependent recombination at dif, as judged by growth competition (Bigot et al., 2004). Competition was assessed at 10 generation intervals between the strains (Km<sup>R</sup> or Tp<sup>R</sup>) expressing the indicated XerD variant and wild-type XerD from appropriate plasmids in a chromosomal XerD<sup>–</sup> background. A complementary competition experiment in which the chromosomal antibiotic resistance markers were exchanged led to <br>the relative proportions of the two strains were assessed every 10 generations by comparing viable counts on Ap agar with those on Km Ap. The XerD<sup>+</sup> strain out-competed the XerD<sup>–</sup> strain as expected, while the XerD quadruple substitution was almost as poor in competition as the XerD<sup>–</sup> strain. These results correlate well with the plasmid resolution data and reinforce our conclusion that the XerD[RQ.QQ] region carries major determinants for interaction with FtsK<sub>Ec</sub>, rather than for core recombinase activity.<br><br>Mapping regions of γ subdomain that interact with XerD<br>Species specificity in FtsK<sub>C</sub>–XerCD interactions in vivo (Yates et al., 2003) was also demonstrated in vitro in BSN cleavage reactions (Fig. 8A). E. coli XerD-mediated cleavage of BSN is efficiently stimulated in reactions containing Ec XerCD and Ec MBP<sub>γ</sub>, but not in reactions containing Hi MBP<sub>γ</sub>. Similarly, Hi MBP<sub>γ</sub>, but not Ec MBP<sub>γ</sub>, could stimulate cleavage of BSN by Hi XerCD. In order to gain some insight into where the possible determinants of interaction with XerD are located in γ<sub>2</sub>, we made comparison of the γ<sub>2</sub> regions from Ec and Hi FtsK<sub>Ec</sub> (Fig. 8B). This comparison revealed a short region of amino acid sequence divergence, TEKRKA, in Ec FtsK, predicted to lie mainly in a loop between two sequence-conserved α-helices. A more detailed comparison of this
A. Species specificity of the FtsKγ–XerCD interaction between E. coli and H. influenzae in vitro. The indicated 60 min reactions were analysed by 0.1% SDS-6% PAGE.

B. Amino acid sequence comparison between the γ regions of E. coli and H. influenzae. The elements of the Ec FtsKγ secondary structure derived from structure predictions are shown above the sequence; α-helices as horizontal grey lines, β-strand as a black line. The region of most notable sequence divergence is shown in bold font.

C. FtsKγ variants were tested for binding to immobilized Ec HisXerD (open bars) and for their ability to stimulate diff-BSN DNA cleavage mediated by XerD (grey bars). The results of both assays are expressed as a percentage of the Ec MBPγ activity (100%).

D. Paradigm of Cre–loxP recombination as applied to the activation of XerD by FtsKc. In a tetrameric recombination complex, only two of the four recombinase molecules are in an active state at any given time (either XerC or XerD in the case of XerCD). In the absence of FtsKc, XerCD–diff forms an ‘XerC-active’ synaptic complex preferentially (Hallet et al., 1999). In this conformation, XerC is active as a consequence of its C-terminal donor region interacting within the acceptor region of an XerD molecule bound to the same duplex; this positions the catalytic tyrosine of the active XerC molecule adjacent to the scissile phosphate (view from the C-terminal side of the complex). The switch from the ‘XerC-active’ to the ‘XerD-active’ configuration in a heterotetramer requires either dissolution of all recombinase–recombinase interactions and reformation of new interactions that lead to an altered DNA conformation, or dissociation of each DNA duplex from one of its recombinase binding sites and reformation of a complex with altered DNA paths. The action of FtsKc could be through pathway a, in which it directs the formation of the XerD-active state on duplex, and/or through pathway b, where the heterotetramer is the substrate for FtsKc action.

New insight into FtsKc-dependent chromosome dimer resolution

In the work reported here, we have initiated dissection of a highly conserved interaction between a recombination machine and a DNA translocase, a reaction that plays a central role in the late stages of bacterial chromosome segregation. In particular, we have characterized the specific interaction that leads to activation of the XerD recombinase. The recognition between a small region of the FtsKc γ subdomain and a region close to the C-terminus of XerD is necessary for the stimulation of BSN cleavage and for their ability to stimulate cleavage of BSN by Ec XerD (Fig. 8C). The results show that replacement of TEKRKA of Ec MBPγ by the Hi sequence, INTGTT, resulted in almost complete loss of interaction with Ec XerD, with the activity of this variant being similar to that of Hi MBPγ. Variants MBPγ[K1279T] and MBPγ[E1278N] had similar activities as Ec MBPγ (not shown), while variants MBPγ[K1279T] and MBPγ[R1280G] showed slightly reduced activity. Ec MBPγ[K1281T] had moderately reduced activity, while Ec MBPγ[A1282T] activity was dramatically reduced, being similar to Hi MBPγ. We conclude that the ‘KRKA’ C-terminal region of XerD is involved in the FtsKc–XerD interaction and that the strength of this interaction correlates well with the ability to stimulate cleavage of BSN by XerD.

region among γ-proteobacteria supported the view that this region might interact with XerD (not shown).

Therefore, Ec MBPγ variants in which whole or parts of the TEKRKA sequence were substituted by amino acids from the Hi region were constructed. These variants were assayed for their ability to bind immobilized Ec HisXerD and for their ability to stimulate cleavage of BSN by Ec XerD (Fig. 8C). The results show that replacement of TEKRKA of Ec MBPγ by the Hi sequence, INTGTT, resulted in almost complete loss of interaction with Ec XerD, with the activity of this variant being similar to that of Hi MBPγ. Variants MBPγ[T1277I] and MBPγ[E1278N] had similar activities as Ec MBPγ (not shown), while variants MBPγ[K1279T] and MBPγ[R1280G] showed slightly reduced activity. Ec MBPγ[K1281T] had moderately reduced activity, while Ec MBPγ[A1282T] activity was dramatically reduced, being similar to Hi MBPγ. We conclude that the ‘KRKA’ C-terminal region of XerD is involved in the FtsKc–XerD interaction and that the strength of this interaction correlates well with the ability to stimulate cleavage of BSN by XerD.

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by XerD, and for the reaction of such a complex with intact linear *dif* duplex in the absence of ATP binding or hydrol-
ysis. This interaction is functionally relevant because its
impairment leads to the *in vivo* loss of XerCD recombina-
tion at *dif* and the concomitant loss of chromosomal dimer
resolution. As yet we do not know whether this interaction
alone is sufficient for FtsKγ to activate XerD, although all
of the determinants for the interaction reside in the 62-
amino-acid γ subdomain.

The structures of a whole range of intermediates in
Cre– *loxP* recombination (Gopaul and Van Duyne, 1999)
provide a paradigm that can be applied to FtsKγ–depen-
dent XerCD recombination at *dif* (Fig. 8D). In this para-
digm, the heterotetrameric synaptic complex can adopt
one of two configurations, ‘XerC-active’ (bottom), or ‘XerD-
active’ (right). The active recombinase has its C-terminal
‘donor’ region engaged into a specific ‘acceptor’ region of
its partner recombinase bound to the same duplex. This
positions the catalytic tyrosine of the active recombinase
close to the scissile DNA phosphodiester. Donor–accept-
or interactions between recombinases bound to the dif-
f erent duplexes participate in synopsis. In the absence of
FtsKγ, the XerCD–*dif* heterotetrameric complex preferen-
tially adopts the ‘XerC-active’ configuration when the
duplex DNA is intact. In order for the ‘XerD-active’ config-
uration to be adopted, FtsKγ must convert a ‘XerC-active’
heterotetramer to the ‘XerD-active’ heterotetramer through a
remodelling reaction (pathway b), and/or stimu-
late the formation of the ‘XerD-active’ state on duplex
(pathway a), two such duplexes forming a ‘XerD-active’
heterotetramer. The latter pathway could occur before
synapsis or after a ‘XerC-active’ synaptic complex has
been dissociated by FtsKγ action. In either scenario, we
propose that the specific interaction of FtsKγ with XerD
identified here promotes formation of the ‘XerD-active’
state. Formally, this could be achieved by compromising
the interaction that leads to XerC being active in the
absence of FtsKγ and/or facilitating the interaction that
makes XerD active. Our data are most easily accommo-
dated in a model in which the interaction of the C-terminus
of XerD with an acceptor region in XerC is facilitated, as
the C-terminal region of XerD, identified as being neces-
sary for the interaction with FtsKγ, is more likely to com-
prise part of the putative XerD donor region than the XerD
acceptor region (Hallet *et al.*, 1999). Because of the
expected intimate association of XerD with XerC in a
synaptic complex, it is not impossible that FtsKγ might
additionally interact, although weakly, with XerC dur-
ing the formation of the ‘XerD-active’ state. Examination of the
Cre– *loxP* structures suggests that a recombinase C-ter-
minal donor region docked with the acceptor region of a
partner recombinase would have the potential to interact
with a surface-located peptide ligand present in the γ
subdomain.

The ability of FtsKγ derivatives to stimulate the forma-
tion of an ‘XerD-active’ synaptic complex on BSN is facil-
itated by the bottom strand nick present in BSN, which
allows formation of some ‘XerD-active’ heterotetrameric
state in the absence of FtsKγ. Presumably DNA flexibility
arising from the nick allows the ‘XerD-active’ conformation
to form, thereby relieving the requirement for ATP-depen-
dent remodelling by FtsKγ. We do not know whether
FtsKγ, in addition, stimulates catalysis by XerD once the
‘XerD-active’ synaptic complex has formed; we have no
evidence to support the existence of such a second step.

The conversion of an ‘XerC-active’ heterotetrameric com-
plex, which forms readily in the absence of FtsKγ, to an
‘XerD-active’ complex requires either the dissolution of all
initial recombinase–recombinase interactions and the cre-
anction of new ones, or a switch in the path of DNA in the
synaptic complex that would require recombinase–DNA
interactions be broken and re-made (Fig. 8D). Whether
this would require a complete dissociation of the heter-
etetrameric complex into two separated duplexes is
unclear. We imagine that when the FtsKγ γ subdomain
fragment interacts with XerD bound to BSN, a single γ
determinant interacts with a single XerD molecule,
although we do not yet know whether both XerD mole-
cules in a heterotetrameric BSN complex need to undergo
such interactions for stimulation of cleavage by XerD. With
wild-type multimeric FtsKγ multiple γ subdomains are
potentially available for binding to the recombinases.

Future experiments need to address precisely how
interaction of FtsKγ with XerCD leads to recombination
activation in a two-duplex heterotetrameric complex, how
DNA sequence-directed FtsKγ translocation facilitates the
simple synopsis of distant *dif* sites, and how FtsKγ
switches from being a DNA translocase to a nucleoprotein
remodelling machine once it encounters XerCD bound to
*dif*-DNA.

**Experimental procedures**

**Bacterial strains**

*In vivo* assays of XerCD and FtsKγ function were carried out in *Ec* AB1157 derivatives (Bachmann, 1972). DS9008 and DS9028 are XerDγ derivatives marked by KmR and TpR respectively (Blakely *et al.*, 1993; D.J. Sherratt, unpublished).

**Recombinant proteins**

Recombinant proteins were produced using standard meth-
ods. MBP fusions to FtsKγ fragments were constructed by
PCR amplifying the desired region of *ftsK* and cloning into
the pMAL-c2x vector (NEB) using EcoRI and HindIII restric-
tion sites. Amino acid substitutions in the *ftsK* vector (NEB) using EcoRI and HindIII restric-
tion sites. Amino acid substitutions in *Ec* MBPγ and *Ec* XerD were obtained by site-directed mutagenesis using mutagenic
primers. *Ec* XerCD, XerCD chimeras, FlFtsKγ and HiXerCD were purified as described previously (Subramanya *et al.*, 2006).
1997; Ferreira et al., 2003; Aussel et al., 2002; Yates et al., 2003). His-tagged Ec XerC and XerD, XerD deletions and XerD point mutants were affinity purified on nickel resin followed by chromatography on a heparin column. His-tagged FtsK_{50C} was purified on nickel resin followed by heparin and DEAE columns. MBP-FtsKy variants were purified on amylose resin and concentrated in spin concentrators.

**Intermolecular recombination and suicide substrate assays**

A 425 bp, unlabelled, dif-containing substrate was produced by PCR on pMIN33 (Blakely et al., 1993). Short linear or nicked, dif-containing, DNA fragments were produced by annealing appropriate oligonucleotides, followed by purification by polyacrylamide gel electrophoresis (PAGE). The longest strand was ^32P-labelled at the 5′ end before annealing. BSNe was constructed by ligating a 200 bp fragment to BSN; its radiolabel was at the same position as that in BSN. Recombination products were carried out in 10 µl of reaction buffer (10 mM Tris-HCl pH 7.5, 1 mM DTT, 15 mM MgCl₂) and contained 0.5 mg ml⁻¹ BSA, 60 mM NaCl and 8% glycerol, 2.5 mM ATP, 2 nM labelled DNA, 2 nM unlabelled DNA, 500 nM XerC, 250 nM XerD, 500 nM FtsK_{50C} or 1–5 µM MBP-FtsKy derivatives. Cleavage of suicide substrates used the same conditions other than that unlabelled DNA was absent, except in the experiment shown in Fig. 4. FtsK_{50C} or MBP-FtsKy derivatives were added last to start the reactions. Reactions were incubated at 37°C for up to 60 min. Products were analysed by 0.1% SDS, 4% or 6% PAGE in Tris-borate buffer (TBE). Gels were scanned and quantified using a Fuji FLA3000 fluorimager and ImageQuant software.

In experiments designed to test whether synopsis of two duplexes is required for the action of FtsK, a constant amount of radiolabelled TSN or BSN DNA (5 nM) was mixed with increasing concentrations of the same unlabelled DNA fragment (overall concentration range 5–45 nM). Poly(dI-dC)-poly(dI-dC) was added to a final concentration of 125 µg ml⁻¹ and sufficient XerCD and FtsK_{50C} were added to ensure saturation at the highest DNA concentration.

**Physical interaction assay**

His-tagged proteins were loaded and immobilized on cobalt chelated affinity agarose (Pierce) in 25 mM Tris-HCl pH 7.2, 150 mM NaCl and 40 mM imidazole. After series of washes to remove any unbound His-tagged protein, the untagged protein of interest was loaded. Proteins that were not retained due to physical interaction with His-tagged protein were removed in the subsequent washing steps. Interaction was assessed on 0.1% SDS-10% PAGE gels following a selective co-elution of the interacting proteins by 300 mM or 400 mM imidazole. The amounts of proteins in samples were determined by staining the gel with SYPRO Orange, followed by scanning in a Fuji FLA 3000 fluorimager and subsequent quantification.

**In vivo plasmid resolution assay**

An *E. coli* strain deleted for xerD (DS9028) was co-transformed with plasmid carrying wild-type xerD gene or its variant, expressed from the *plac* promoter (pRM132, Ap⁺; Blakely et al., 1993), and with a low copy number reporter plasmid, containing either a dif-Km⁻-dif cassette (pFX142, Sp⁺; Aussel et al., 2002) or a psi-Km⁻-psi cassette (pzi218, Sp⁺). Transformants were plated on LB agar supplemented with Km, Ap and glucose (1%). A population of approximately 20 colonies was inoculated into LB Sp, Ap liquid culture and grown to A₆₀₀ of 0.4, induced with IPTG (100 µM) and grown overnight. Plasmid DNA was extracted and separated on 1% agarose-TAE gels, stained with SybrGreen and scanned on a Fuji FLA3000 fluorimager. Resolution was quantified by transformation of DNA samples into a Xer⁺ strain and determination of the fraction of Sp⁵ transformants that are Km⁺.

**Co-culture competition assay**

This assay is used to assess the efficiency of FtsK⁺-dependent chromosome dimer resolution (Pérals et al., 2000; Bigot et al., 2004). A pair of strains, each lacking a functional xerD gene (DS9008, Km⁺ and DS9028 Tp⁺) were transformed with a pUC-derived plasmid carrying either a wild-type xerD gene or xerD variant under *plac* promoter control. Cells were grown in LB Ap until A₆₀₀ = 0.4, induced with IPTG (100 µM), mixed 1:1, diluted and grown in serial cultures for 40 generations. The relative ratios of colony-forming units of both strains in cultures were assessed at 10 generations time points by plating on LB Ap Km agar and LB Ap agar.

**Acknowledgements**

We thank Greg Van Duyne for stimulating discussions and Katie Christoffers for technical assistance. This work was supported by the Wellcome Trust. J.Y. was supported by a MRC studentship for postgraduate training and I.Z. by a Heilmore postgraduate award of Oxford University.

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