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The DNA repair helicase UvrD is essential for replication fork reversal in replication mutants

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Replication forks arrested by inactivation of the main *Escherichia coli* DNA polymerase (polymerase III) are reversed by the annealing of newly synthesized leading- and lagging-strand ends. Reversed forks are reset by the action of RecBC on the DNA double-strand end, and in the absence of RecBC chromosomes are linearized by the Holliday junction resolvase RuvABC. We report here that the UvrD helicase is essential for RuvABC-dependent chromosome linearization in *E. coli* polymerase III mutants, whereas its partners in DNA repair (UvrA/B and MutL/S) are not. We conclude that UvrD participates in replication fork reversal in *E. coli*.

Keywords: DNA replication; DNA repair; *Escherichia coli*; DNA recombination

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INTRODUCTION

Fidelity in DNA replication is required to maintain genome integrity. In most organisms, genome rearrangements arise when stalled DNA replication forks are processed inappropriately (Carr, 2002; Kolodner *et al*, 2002). Depending on the cause of replication arrest, different strategies are used to restart blocked replication forks. In *Escherichia coli*, the replisome is composed of a dimer of polymerase III holoenzyme (Pol III HE), the DnaB helicase and the DnaG primase. Replication forks blocked by a partial or total inactivation of the DNA polymerase, or by inactivation of the replicative helicase, undergo a specific reaction named replication fork reversal (Flores *et al*, 2001; Michel *et al*, 2001; Grompone *et al*, 2002). This reaction involves the annealing of the newly synthesized leading and lagging strands and the concurrent pairing of the template strands, which results in the formation of a four-arm double-strand DNA junction with a DNA double-strand end (Fig 1A). The enzyme that acts at DNA double-strand ends in *E. coli* is the exonuclease/helicase RecBCD, which degrades linear DNA up to a specific sequence named *chi* at which it promotes RecA polymerization (Kuzminov, 1999). The processing of reversed forks is well characterized (Seigneur *et al*, 1998; Grompone *et al*, 2002): the DNA double-strand end is processed by RecBCD (Fig 1B), which either recombines it (Fig 1C) or degrades it (Fig 1D), and the four-way junction is recognized by RuvABC (Fig 1B), a complex known to resolve Holliday junctions formed by homologous recombination (for a review, see West, 1997). Regardless of whether the DNA double-strand end at reversed forks is processed by homologous recombination (Fig 1B,C) or by DNA degradation (Fig 1B–D), the resulting three-way DNA structure is a target for the reassembly of a functional replisome, and replication can restart (Marians, 2000; Flores *et al*, 2002). In the absence of processing of the DNA double-strand end (absence of RecBC), RuvABC-catalysed resolution of the Holliday junction formed at blocked forks causes chromosome linearization and cell death (Seigneur *et al*, 1998; Fig 1E).

So far, the initial step of reversed fork formation, the annealing of leading and lagging strands, is only understood in the *dnaBts* mutant, where formation of a RuvABC substrate following replication inactivation requires the presence of a functional RecA protein. It was proposed that the absence of DnaB allows RecA-mediated annealing of the blocked leading and lagging strands (Seigneur *et al*, 2000). In contrast, the reaction occurs in the absence of RecA, hence by a different pathway, in the three Pol III HE mutants that were studied: *dnaEts* impaired for the Pol III catalytic subunit, *dnaNts* impaired for the β-clamp (the processivity factor) and *holD* impaired for one of the subunits of the clamp loader complex (Flores *et al*, 2001; Grompone *et al*, 2002).

UvrD (also called helicase II) is a dimeric helicase that belongs to the helicase superfamily I (Gorbalenya & Koonin, 1993). *In vitro*, UvrD translocates unidirectionally in a 3′ to 5′ direction and, at low concentration, it prefers unwinding DNA with a 3′ DNA single-strand overhang (Matson, 1986). However, at a higher protein concentration, UvrD can also unwind DNA from a nicked substrate or from a blunt end (Runyon *et al*, 1990). In addition, UvrD can also unwind RNA:DNA hybrids (Matson, 1989). UvrD is essential (i) for nucleotide excision repair (NER), as it removes the oligonucleotide produced by UvrC-catalysed DNA cleavage on both sides of a lesion, and (ii) for mismatch repair, as it removes the DNA fragment carrying the misincorporated nucleotide after

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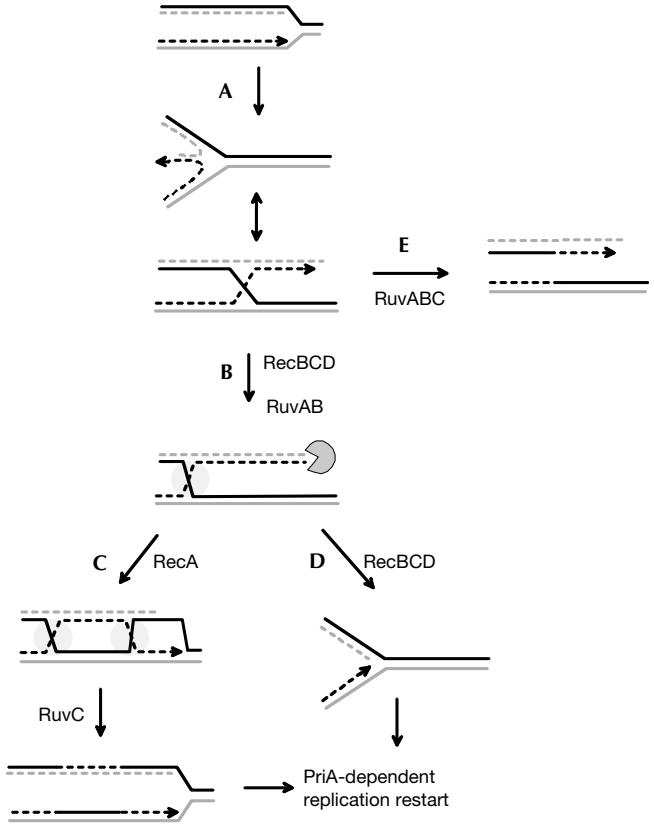

Fig 1 | The replication fork reversal model (adapted from Seigneur *et al*, 1998; Michel *et al*, 2001). In the first step (**A**), the replication fork is arrested by impairment of a replication protein, causing fork reversal. The reversed fork forms a four-armed structure (Holliday junction, two alternative representations of this structure are shown, open X and parallel stacked X). In Rec+ cells (**B,C**), RecBCD initiates RecA-dependent homologous recombination at a *chi* site present on the DNA double-strand end and the two Holliday junctions (one formed by reversal, one by homologous recombination) are resolved by RuvABC. Alternatively, if RecBCD encounters the Holliday junction before encountering *chi*, or in the absence of RecA (**B–D**), the DNA double-strand end is degraded up to the Holliday junction, restoring a fork structure. In both cases, replication restarts by a PriA-dependent process. In the absence of RecBCD (**E**), resolution of the Holliday junction by RuvABC causes chromosome linearization. Continuous lines: parental chromosome; dashed lines: newly synthesized strands; circle: RuvAB; incised circle: RecBCD.

cleavage by the MutH endonuclease (Lahue *et al*, 1989; Dao & Modrich, 1998). UvrD is thought to interact with its partner repair proteins: it is loaded by MutL at the nicked GATC sequence during mismatch repair and it was proposed to dislodge the UvrABC complex from the incised DNA during NER (Ahn, 2000; Mechanic *et al*, 2000). In addition, UvrD has a dual role in homologous recombination: it participates in homologous recombination initiated by RecFOR in *recBC sbcBC* mutants (Mendonca *et al*, 1993; Washburn & Kushner, 1993) and, conversely, it acts as an anti-recombinase *in vitro* and *in vivo* (Zieg *et al*, 1978; Morel *et al*, 1993; Bierne *et al*, 1997; Petranovic *et al*, 2001). Several observations suggest that UvrD may be involved in DNA replication. UvrD stimulates DNA synthesis by DNA Pol III at an artificial replication fork *in vitro* (Kuhn & Abdel-Monem, 1982), and is regularly found in preparations of the DNA Pol III HE (Lahue *et al*, 1989). UvrD is essential for the viability of cells that lack Rep, a replicative helicase (Washburn & Kushner, 1991), and for the replication of rolling circle plasmids (Bruand & Ehrlich, 2000).

We studied here replication fork reversal in the *dnaEts* and *dnaNts* mutants. We report that the UvrD protein is essential for the formation of reversed forks, providing an unexpected and crucial role for this protein in response to replication arrest.

RESULTS

DNA breakage requires UvrD in *dnaNts recBCts*

The replication mutants that undergo replication fork reversal suffer RuvABC-dependent chromosome linearization in the absence of RecBCD because the Holliday junctions formed by fork reversal are then resolved by RuvABC without repair of the DNA double-strand end (Fig 1E). We used the combination of two thermosensitive mutations, *recBts* and *recCts* (designated *recBCts* hereafter), to inactivate the RecBC enzyme at 37 and 42 °C. These two *recBCts* mutations were combined with the *dnaNts* mutation that inactivates the Pol III β-clamp at high temperature. We quantified chromosome breakage by pulse field gel electrophoresis (PFGE) at three different temperatures: 30, 37 and 42 °C, which are permissive, semipermissive and nonpermissive temperatures for the *dnaNts* mutant. As previously reported, chromosome breakage occurred only at semipermissive temperature in the *dnaNts* mutant (37 °C), suggesting that replication fork reversal requires a β-clamp function (Grompone *et al*, 2002; Fig 2A). To determine whether UvrD is involved in replication fork reversal in the *dnaNts* mutants, a *uvrD* null mutation was introduced in a *dnaNts recBCts* mutant and linear DNA formation was quantified. The absence of UvrD protein caused a significant decrease in the amount of linear DNA formed after partial inactivation of DnaN at 37 °C, to the level observed in a DnaN+ *recBCts uvrD* mutant (Fig 2A). As expected, the introduction of the wild-type *uvrD* gene on a plasmid restored the original amount of linear DNA (whereas the vector plasmid pGB2 had no effect), indicating that the decrease in linear DNA in the *uvrD* mutant results from the absence of a functional UvrD protein (Fig 2B).

The very low viability of the *uvrD ruv* double mutant prevented us from testing whether the remaining linear DNA in the *dnaNts recBCts uvrD* mutant requires RuvABC for its formation (data not shown). The need for RuvABC proteins for the full viability of a *uvrD* null mutant may result from the increased level of homologous recombination associated with *uvrD* inactivation (five- to tenfold). Considering that at least 50 copies of the RuvABC complex are present in a RuvABC+ strain (West, 1997), it is unlikely that the decrease of chromosome linearization following inactivation of the *uvrD* gene results from a lack of RuvABC proteins (due to the binding of RuvABC complexes to recombination intermediates). Nevertheless, to rule out fully this possibility, we measured the level of linear DNA in the *dnaNts recBCts uvrD* mutant in the presence of about ten additional copies of functional *ruvABC* genes provided from a plasmid (Fig 2B, pGB-*ruvABC*). Increasing the number of copies of *ruvABC* genes did not modify the level of linear DNA formation in *dnaNts*

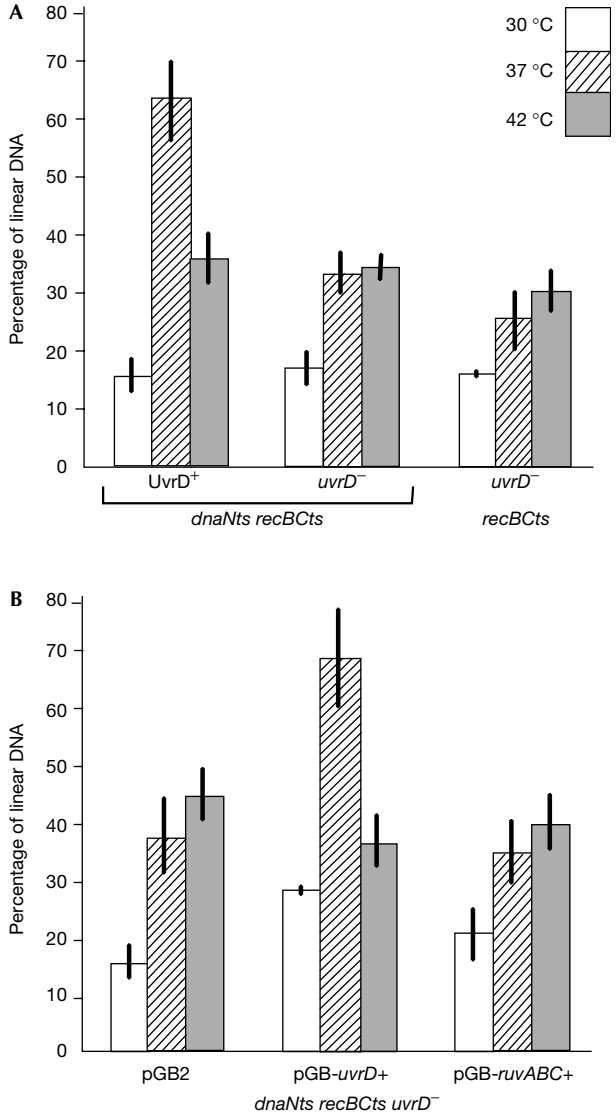

Fig 2 | Inactivation of the UvrD protein prevents chromosome linearization in the *dnaNts recBCts* mutant. The histograms indicate the percentage of linear DNA in cultures propagated either at permissive temperature (30 °C, white blocks), or for 3 h at semipermissive temperature (37 °C, hatched blocks), or restrictive temperature (42 °C, grey-shaded blocks). Bold lines indicate the standard deviation. The genotypes of mutants used are indicated below the blocks. (**A**) The large amount of linear DNA in the *dnaNts recBCts* strain at 37 °C is decreased by the *uvrD* null mutation, to the level of linearization observed in DnaN$^+$ conditions. The *dnaNts recBCts* and the *dnaNts recBCts uvrD* strains are highly significantly different at 37 °C ($P<0.001$). The three strains are not significantly different at 42 °C, and *dnaNts recBCts uvrD* and *recBCts uvrD* strains are not significantly different at 37 °C ($P>0.2$). (**B**) The formation of linear DNA at 37 °C is restored by introduction of the *uvrD*$^+$ gene on a plasmid. Introduction of the vector plasmid pGB2 or of extra copies of the *ruvABC* gene has no effect. The increase in linear DNA at 37 °C in the presence of pGB-*uvrD* is highly significant compared with pGB2- and pGB-*ruvABC*-containing strains ($P<0.001$). pGB-*ruvABC* has no significant effect at 37 °C, and results in all three strains at 42 °C are not significantly different ($P>0.1$).

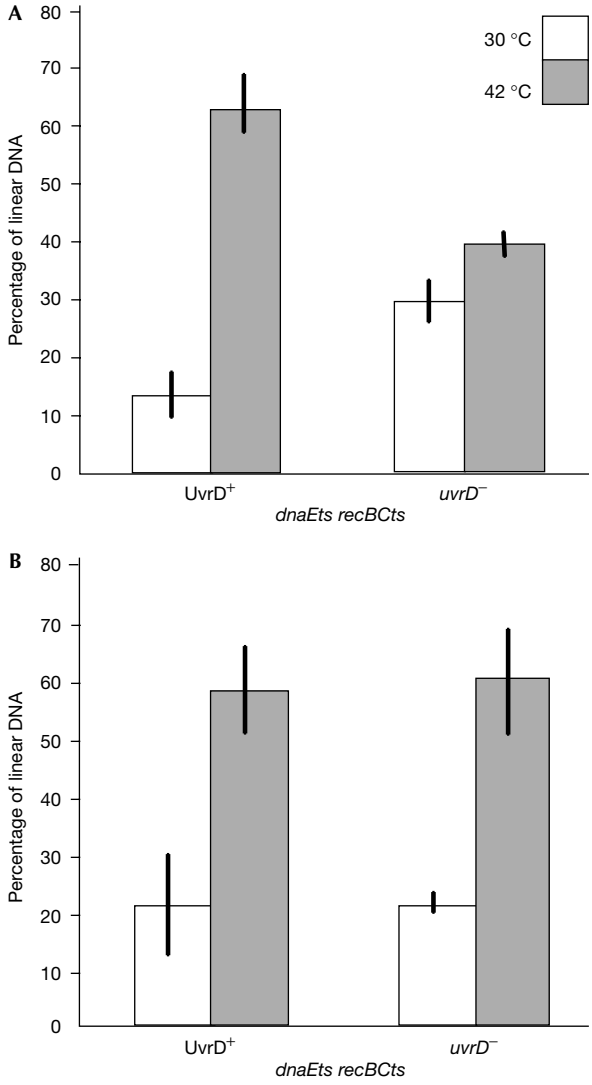

Fig 3 | Inactivation of the UvrD protein prevents chromosome linearization in the *dnaEts recBCts* mutant, but not in the *dnaBts recBCts* mutant. Column shading is the same as in Fig 2. (**A**) The large amount of linear DNA in the *dnaEts recBCts* strain at 42 °C is decreased by the *uvrD* null mutation ($P>0.001$) to the level observed in DnaE$^+$ conditions (see Fig 2; *dnaEts recBCts uvrD* and *recBCts uvrD* are not significantly different at 42 °C, $P>0.1$). (**B**) Inactivation of *uvrD* has no effect in the *dnaBts recBCts* mutant, in which replication fork reversal is RecA dependent.

recBCts uvrD cells at 37 °C, confirming that RuvABC is not limiting in this strain. We conclude that UvrD is required for the formation of the RuvABC substrate following partial inactivation of DnaN in a *recBC* mutant context.

DNA breakage requires UvrD in *dnaEts recBCts*

Replication fork reversal also occurs following inactivation of the *dnaE* gene, which encodes the polymerase catalytic subunit of Pol III HE (Grompone *et al*, 2002). We constructed a *dnaEts recBCts uvrD* mutant and measured chromosome linearization. The level of linear DNA formed after inactivation of the polymerase DnaE at

42 °C was significantly reduced in the absence of UvrD (Fig 3A). Complementation of the *uvrD* mutation in the *dnaEts recBCts uvrD* strain by introduction of a functional *uvrD* gene on a plasmid (pGB-*uvrD*) restored about 60% of DNA breakage (not shown).

The viability of the *dnaEts* and *dnaNts* mutants at 37 °C was previously shown to be strongly decreased in a *recBC* null background, which correlates with the formation of linear chromosomes (Grompone *et al*, 2002). Similarly, the inactivation of *uvrD* affected the viability of *dnaEts* and *dnaNts* mutants at 37 °C (data not shown), suggesting that UvrD has an essential role when replication is impaired by a Pol III HE mutation.

In contrast with the Pol III mutants studied here, replication fork reversal requires RecA in the helicase mutant *dnaBts* and was therefore proposed to occur by RecA-catalysed annealing of the blocked leading and lagging strands (Seigneur *et al*, 2000). Accordingly, the inactivation of *uvrD* had no effect on the level of linear chromosomes formed after DnaB inactivation in a *dnaBts recBCts* mutant (Fig 3B).

Inactivation of NER or mismatch repair

UvrD is required for the repair of UV lesions by NER and for mismatch repair. We tested whether the repair proteins that act with UvrD in NER (UvrA, UvrB) or in mismatch repair (MutL, MutS) have a role at inactivated replication fork in the *dnaEts* or *dnaNts* mutants. The *uvrA, uvrB, mutL* or *mutS* mutations were transferred to the *dnaEts recBCts* and *dnaNts recBCts* mutants and linear DNA formation was measured (supplementary Fig S1 online). The amount of linear DNA formation was not significantly modified when NER was inactivated by a *uvrA* or *uvrB* mutation, or when mismatch repair was inactivated by a *mutL* or *mutS* mutation, or when both were inactivated (supplementary Fig S1 online and Fig 4; except for a slight increase at 42 °C in the *dnaNts recBCts uvrA* and in the *dnaNts recBCts uvrB* strains, see supplementary Fig S1 online). In Pol III$^+$ cells, inactivation of *uvrA* and/or *mutL* did not increase linear DNA formation in a *recBCts* background, confirming that the linear DNA formed in *dnaNts recBCts uvrA mutL* or *dnaEts recBCts uvrA mutL* mutants after a shift to a high temperature results from replication inhibition (data not shown). Furthermore, in the *dnaNts recBCts* mutant that lacks both UvrA and MutS (*dnaNts recBCts uvrA mutS* mutant), formation of the linear DNA (i) requires the presence of UvrD (confirming that UvrD acts at blocked forks in the absence of both UvrA and MutS) and (ii) requires RuvABC (confirming that the chromosome linearization occurs through the formation of a RuvABC substrate in these mutants; Fig 4). We conclude that UvrD is required for chromosome breakage in the absence of NER and mismatch repair proteins.

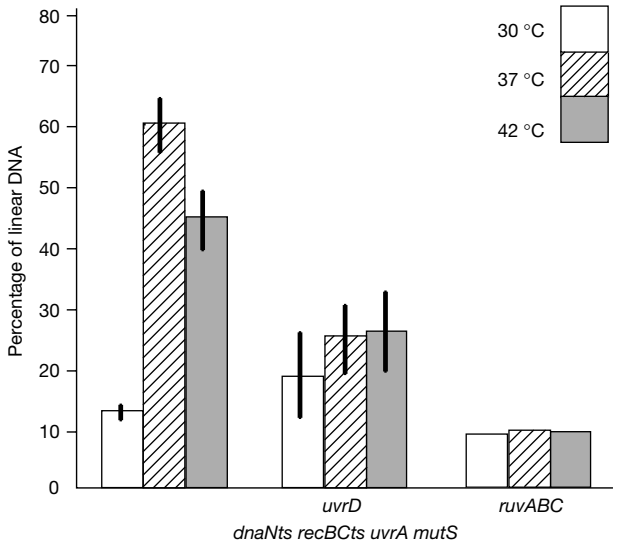

Fig 4 | In a *dnaNts recBCts* mutant that lacks both NER (*uvrA* mutation) and mismatch repair (*mutS* mutation), chromosome linearization is still dependent on UvrD and RuvABC. Symbols are as in Fig 2. In a *dnaNts recBCts uvrA mutS* strain, inactivation of *uvrD* or *ruvABC* causes a highly significant decrease in the amount of linear DNA at 37 and 42 °C ($P > 0.001$).

Inactivation of the helicase function of UvrD

To test whether the helicase function of UvrD is required for replication fork reversal, we used a previously characterized mutation in the helicase motif IV of the chromosomal *uvrD* gene (R284A). The purified mutated protein UvrD-R284A is severely compromised for ATP binding and for unwinding activity, and a Δ*uvrD* mutant in which the UvrD-R284A protein is expressed from a plasmid is deficient for both NER and mismatch repair (Hall & Matson, 1997; Zhang *et al*, 1997). We observed that a strain carrying the *uvrD*-R284A mutation in the chromosome was similarly deficient for both types of DNA repair (JJC2643; supplementary Table S1 online, see supplementary information online for strain construction; data not shown). Our attempts to introduce the *dnaNts* allele in this mutant failed, indicating that the helicase-deficient *uvrD*-R284A allele is dominant negative at 30 °C in a *dnaNts* context. *dnaEts* could be introduced in a *uvrD*-R284A mutant at 30 °C (JJC2683; supplementary Table S1 online) but not in a *uvrD*-R284A *recBCts* strain, precluding the study of DNA breakage (data not shown). A dominant-negative effect of a UvrD protein mutated at the invariant Lys residue of the Walker A motif was previously reported in *E. coli*, suggesting a deleterious effect of UvrD proteins able to bind DNA but unable to act (George *et al*, 1994). Although the *uvrD*-R284A mutation did not seem to be deleterious at 30, 37 or 42 °C in an otherwise wild-type background, the lethality conferred by this mutation at 30 °C in *dnaNts* and *dnaEts recBCts* strains suggests that Pol III is slightly defective in these strains at permissive temperature and supports the view that UvrD acts at such inactivated replication forks.

DISCUSSION

In this work, we show that the helicase UvrD is essential for replication fork reversal in two *E. coli* Pol III mutants, impaired for the catalytic subunit of Pol III (DnaE) or for the β-clamp (DnaN). *E. coli* encodes more than ten helicases known to act in various DNA or RNA transactions, or of unknown function. In addition to UvrD, several other *E. coli* helicases were tested for their putative role in replication fork reversal. We previously showed that inactivation of RecQ or of the helicase function of PriA did not affect replication fork reversal (Grompone *et al*, 2002). We also observed that inactivation of helicase IV (encoded by the *helD* gene) or of DinG (an SOS-inducible helicase of unknown function; Voloshin *et al*, 2003) also had no effect (M.J. Flores,

B. Michel, unpublished data). These observations point to a specific role of UvrD in replication fork reversal.

Our results indicate that UvrD has a crucial role after replication inactivation by participating in the first step of fork reversal (Fig 1A). The existence of at least two different pathways for fork reversal, RecA dependent in *dnaBts* and UvrD dependent in Pol III mutants, suggests that the accessibility of different proteins to the replication fork may be determined by the nature of the replication block, either by the structure of DNA after replication arrest (extent of synthesis of the leading or lagging strand) and/or by the nature of the polypeptides that remain associated with the inactivated fork.

Several hypotheses can be proposed for the mode of action of UvrD at blocked forks. It could act by itself, by binding to replication forks and unwinding both lagging- and leading-strand ends (it unwinds RNA–DNA hybrids as well as DNA–DNA hybrids and can act from a nick). Alternatively, it could unwind only one strand at the fork and the other one would be unwound by another protein. Finally, in a nonexclusive model, UvrD could remove proteins bound to inactivated replication forks and thereby allow fork reversal catalysed by some other protein(s). The homologue of UvrD in yeast is Srs2, which is suspected to have a role in homologous recombination associated with replication defects. Indeed, in *Saccharomyces cerevisiae,* the Srs2 protein is induced and modified during S phase and *srs2* gene inactivation causes increased genomic instability (Heude *et al*, 1995; Lee *et al*, 1999; Gangloff *et al*, 2000; Liberi *et al*, 2000). The requirement of UvrD for replication fork reversal in *E. coli* opens new fields of investigations to identify the role and the mode of action of the UvrD/Srs2 family of helicases during replication.

METHODS

Strains and plasmids. The strain background is JJC40, which is an *hsdR* Thr$^+$ Pro$^+$ derivative of AB1157 (*leu-6 thi-1, his-4, argE3, lacY1, galK2, ara-14, xylS, mtl-1, tsx-33, rpsL31, supE44*). Details of strain construction and strain genotypes are described in supplementary information online and supplementary Table S1 online.

Measure of linear DNA by PFGE. Quantification of pulse field gels and measures of DNA degradation were performed as previously described (Seigneur *et al*, 1998). Briefly, for chromosome labelling, cells were grown in minimal medium in the presence of tritiated thymidine and deoxyadenosine for 3 h at 30 °C. Then, part of the culture was maintained at 30 °C and part was shifted to 37 or 42 °C for an additional 3 h. Cells were collected, washed and embedded in agarose plugs. Gentle lysis was performed in plugs. Plugs were used for PFGE and the proportion of DNA migrating was determined by cutting each lane in slices and counting the tritium present in the wells and in the gel slices. To avoid DNA damage during PFGE, the apparatus was routinely washed with 0.1% SDS.

The SAS GLM procedure (SAS/STAT version 6.1, Sas Institute Inc., Cary, NC, USA) was used to test the probability that the least-squares means of two strains are equal (type III sum of square, SAS/STAT). Measures are considered as highly significantly different if $P<0.01$, as significantly different if $0.01<P<0.05$ and not significantly different if $P>0.05$.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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