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UvrD helicase, unlike Rep helicase, dismantles RecA nucleoprotein filaments in *Escherichia coli*

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The roles of UvrD and Rep DNA helicases of *Escherichia coli* are not yet fully understood. In particular, the reason for *rep uvrD* double mutant lethality remains obscure. We reported earlier that mutations in *recF, recO* or *recR* genes suppress the lethality of *uvrD rep*, and proposed that an essential activity common to UvrD and Rep is either to participate in the removal of toxic recombination intermediates or to favour the proper progression of replication. Here, we show that UvrD, but not Rep, directly prevents homologous recombination *in vivo*. In addition to RecFOR, we provide evidence that RecA contributes to toxicity in the *rep uvrD* mutant. *In vitro,* UvrD dismantles the RecA nucleoprotein filament, while Rep has only a marginal activity. We conclude that UvrD and Rep do not share a common activity that is essential *in vivo*: while Rep appears to act at the replication stage, UvrD plays a role of RecA nucleoprotein filament remover. This activity of UvrD is similar to that of the yeast Srs2 helicase.

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Introduction

DNA helicases are present in all kingdoms of life, and are probably important in most reactions involving DNA such as replication, repair, recombination and transcription. Their DNA unwinding activities are well characterized at the biochemical and structural levels (for a review, see Delagoutte and von Hippel, 2002a, b). However, much remains to be understood about their roles *in vivo*. In particular, UvrD and Rep are two *Escherichia coli* DNA helicases of the SF1 family that share 40% amino-acid identity, and that are also remarkably similar to the PcrA helicase of Gram-positive bacteria. Some well-defined activities of UvrD, PcrA and Rep have been characterized both *in vivo* and *in vitro*. (i) They participate in the replication of various extragenic elements. The Rep helicase is needed during bacteriophages M13 and ΦX174 replication (Takahashi *et al*, 1979), the UvrD helicase ensures the replication of Gram-negative rolling-circle plasmids (Bruand and Ehrlich, 2000) and the PcrA helicase ensures the replication of Gram-positive rolling-circle plasmids (Petit *et al*, 1998; Anand *et al*, 2004). All these elements share the common characteristic that replication of their leading and lagging strands is uncoupled, and the helicase mediates DNA unwinding ahead of the DNA polymerase during leading strand replication. (ii) UvrD has a role in UV repair where it allows the removal of a 12-nt-long DNA segment containing a UV lesion, after its incision by the combined action of UvrA, UvrB and UvrC (Orren *et al*, 1992). This activity is efficiently complemented by PcrA (Petit *et al*, 1998). (iii) UvrD is involved in mismatch repair, where it promotes the removal of the DNA segment containing the erroneous nucleotide after its incision by the combined action of MutS, MutL and MutH (Modrich, 1994).

Other phenotypes of the *rep, uvrD* and *pcrA* mutants are less well understood: (i) Replication fork progression is twice slower in a *rep* mutant than in the Rep+ strain (Lane and Denhardt, 1975; Colasanti and Denhardt, 1987). (ii) Homologous recombination is increased in a *uvrD* mutant (Zieg *et al*, 1978; Arthur and Lloyd, 1980; Bierne *et al*, 1997a) and decreased in a UvrD overproducing strain (Maples and Kushner, 1982; Petranovic *et al*, 2001). Increased recombination in the *uvrD* mutant could be due to a replication defect, as reported for some replication mutants (Bierne *et al*, 1997b; Flores *et al*, 2001), or to a direct role of UvrD as an antirecombinase as was found *in vitro* (Morel *et al*, 1993). (iii) The *pcrA* single mutant of *Staphylococcus aureus* and of *Bacillus subtilis* is dead (Iordanescu, 1993; Petit *et al*, 1998), as is the *rep uvrD* double mutant of *E. coli* (Taucher-Scholtz *et al*, 1983).

Previously, we took a genetic approach to investigate the essential role of PcrA in *B. subtilis,* and UvrD and Rep in *E. coli*. We reported that the heterologous expression of *pcrA* in the *E. coli rep uvrD* double mutant strain restored its viability (Petit *et al*, 1998). This led to the hypothesis that *pcrA*, and *rep* or *uvrD* shared a common important function *in vivo*. We then found that lethality of the *pcrA* mutant is suppressed by mutations in the *recF*, *recO*, *recL* or *recR* genes of *B. subtilis*. Remarkably, in *E. coli*, lethality of the *uvrD rep* double mutant was also suppressed by mutations in the *recF*, *recO* or *recR* genes (Petit and Ehrlich, 2002).

The key protein for homologous recombination in bacteria, RecA, is loaded onto single-stranded DNA (ssDNA) through the mediation of either one of the two complexes, RecFOR or RecBCD (Kuzminov, 1999). Each complex acts and processes a specific DNA substrate so as to permit RecA binding: RecFOR proteins promote the formation of RecA nucleofilaments on ssDNA gaps covered with SSB (Umezu *et al*, 1993; Webb *et al*, 1997; Morimatsu and Kowalczykowski, 2003). RecBCD enzymatic complex is a double-stranded DNA

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(dsDNA) exonuclease that converts into a recombination enzyme upon encountering a specific DNA sequence, CHI (Kowalczykowski *et al*, 1994). Its entry point into DNA is a double-stranded end. The suppression of helicase mutations lethality by *recF*, *recO* or *recR* mutations suggested a toxic effect due to the RecFOR recombination proteins in the helicase mutants. Two formal possibilities were proposed to explain these data (Petit and Ehrlich, 2002). The helicases may act either during the recombination process to reverse toxic-blocked recombination intermediates, or at a step upstream of recombination, possibly during replication. In the latter case, their absence would allow recombination to occur at a high and toxic level. The question of whether or not Rep and UvrD are acting at the same step in a redundant fashion was left unanswered.

The Srs2 helicase of *Saccharomyces cerevisiae* is the closest found orthologue to UvrD, Rep and PcrA in eukaryotes (Aboussekhra *et al*, 1989). Like the *uvrD* mutant, the *srs2* mutant exhibits a high rate of spontaneous recombination (Aguilera and Klein, 1988). A number of *srs2* mutant phenotypes are suppressed by mutations that prevent formation of Rad51 nucleofilaments. These include UV sensitivity of single *srs2* mutants (Aboussekhra *et al*, 1992) and the synthetic lethality or sickness of certain double mutants involving *srs2*, like the *srs2 sgs1* double helicase mutant (Gangloff *et al*, 2000; Klein, 2001; Fabre *et al*, 2002). These observations led to the idea that one role of Srs2 is to prevent the formation of toxic recombination intermediates by eliminating inappropriate Rad51 nucleofilaments. *In vitro* data showed that Srs2 is indeed able to perform this activity (Krejci *et al*, 2003; Veaute *et al*, 2003).

The suppression of *srs2* phenotypes by mutations in genes controlling the Rad51 pathway is reminiscent of the suppression of the lethality of *pcrA* or *rep uvrD* mutants by mutations affecting the RecF pathway. The aim of this work was to examine the possibility that UvrD, Rep or both helicases act in a way similar to Srs2, by disrupting RecA filaments. An *in vivo* assay based on conjugation, allowing the concomitant measurement of replication and recombination activity, showed that the *rep* and *uvrD* mutants behaved differently, with Rep acting principally on replication and UvrD on recombination intermediates. We also gained evidence that in addition to RecFOR the RecA protein was toxic in the *rep uvrD* mutant. Finally, the effects of the Rep and UvrD helicases on *in vitro* strand exchange assays as well as their ability to disrupt preformed RecA nucleoprotein filaments were studied. We found that UvrD, but not Rep, dismantles the RecA nucleoprotein filament. Our set of results supports the view that the lethality of the *rep uvrD* mutant is not due to a redundant function of these two helicases, but rather that UvrD is essential for the viability of the *rep* mutant. This suggests that toxic RecA filaments are formed in the *rep* mutant, possibly at the fork, and need to be removed by UvrD.

Results

In vivo monitoring of replication and recombination in real time

Because the UvrD and Rep helicases were suspected to affect recombination and/or replication *in vivo*, and because both processes are often intimately connected, an experimental system was set up for concomitant monitoring of replication and recombination as a function of time. It is based on Hfr conjugation, a process during which chromosomal DNA is transferred from a donor to a recipient strain. DNA enters the recipient strain as a single strand starting from a 5′ extremity, and is converted into dsDNA by replication. Replication depends on DNA polymerase III, the main replicative polymerase, and also on DnaB, the replicative helicase (Willetts and Wilkins, 1984; our unpublished data). The role of DnaB is likely to allow primase loading, but nothing is known about the way DnaB itself is loaded. Once replicated, DNA recombines at high rates with the recipient chromosome by homologous recombination (up to 50% of recipient cells may integrate a given allele). To follow replication and recombination in the recipient cell, two slightly different Hfr donors were constructed, and used with recipient strains containing the helicase mutation to be tested.

The replication assay is depicted in Figure 1A. The donor contained a *lacI*^{s} mutation, which encodes a hyper-repressor LacI^s molecule (Willson *et al*, 1964). As a consequence, the donor strain, although harbouring *lacZ*, produces a low level of β-galactosidase in the presence of the IPTG inducer (see Figure 2B, the 'control' curve is Hfr3000 *lacI*^{s}). When the *lacI*^{s}-*lacZ* region of the Hfr donor enters the recipient strain, which is LacZ$^-$, it is first replicated, and then *lacZ* is fully transcribed, for about 1 h (see Figure 2B), the time needed for LacI^s to accumulate and repress *lacZ*. This is designated 'zygotic induction'. During this time period, the β-galactosidase activity of extracts mostly depends on the replication efficiency.

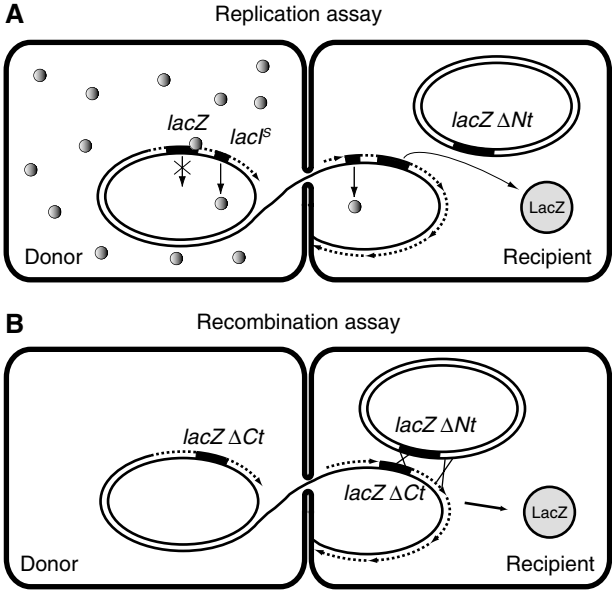

Figure 1 (**A**) Scheme of the replication assay. ssDNA is passing from the donor cell (left side) to the recipient cell (right side), in which it is converted to dsDNA (dotted arrow lines, oriented 5′–3′). In the donor strain (left side), the LacI^s protein (small grey dots) represses strongly the *lacZ* promoter, so that almost no LacZ is produced. In the recipient strain, as soon as the incoming DNA is replicated, a burst of LacZ synthesis occurs (big grey circle), due to the initial absence of LacI^s. After a 1 h delay, sufficient amounts of LacI^s are produced to shut down *lacZ* transcription. (**B**) Scheme of the recombination assay. Recombination occurs between the incoming *lacZΔCt* allele and the *lacZΔNt* allele of the recipient chromosome (black boxes), and leads to a functional gene and enzyme production (big grey circle).

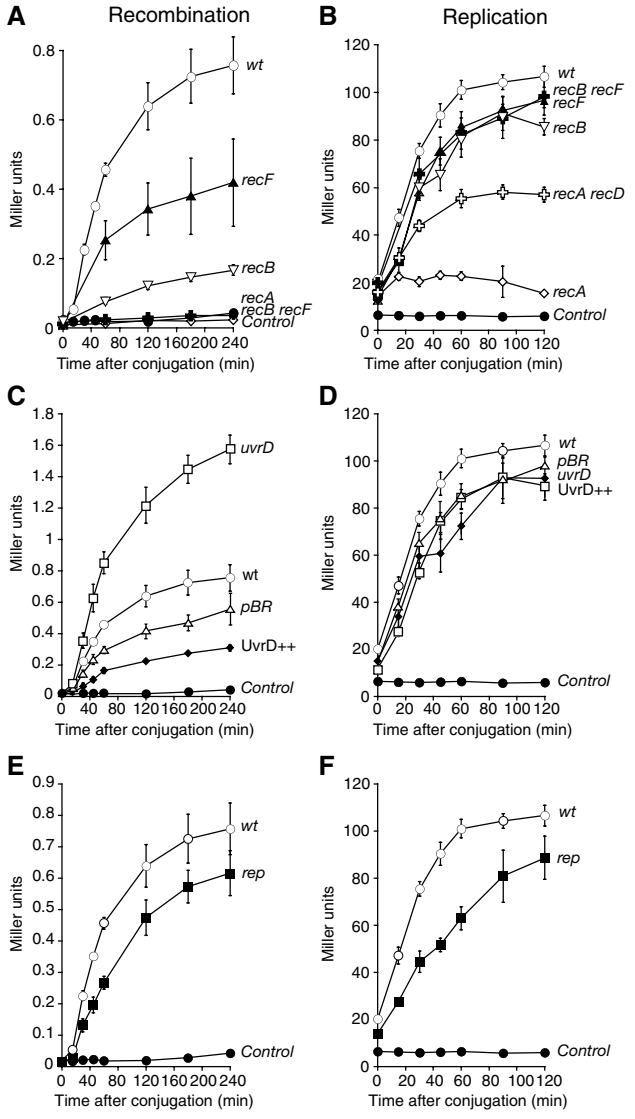

Figure 2 Kinetics of recombination (**A, C, E**) and replication (**B, D, F**) after conjugation in various mutants. Replication and recombination were monitored by using donor strains Nec226 for the replication assay and Nec224 for the recombination assay, as described in the text. All experiments were repeated at least three times. The recipient strains are designated by an alias drawn near each curve: 'wt' Nec223 (○), '*recA*' Nec229 (◇), '*recB*' Nec228 (▽), '*recF*' Nec227 (▲), '*recB recF*' Nec232 (✚), '*recA recD*' Nec230 (✠), '*uvrD*' MAC1058 (□), 'pBR' MAC1068 (Δ), 'UvrD++' MAC1071 (◆) and '*rep*' MAC1065 (■). The 'control' curves in panels A, C and E (●) represent the cross between two identical *lacZΔP* alleles, Nec223 and Nec225 strains, which cannot result in a Lac+ recombinant. The 'control' curve in panels B, D and E represents the Hfr3000 *lacI*^{s} donor strain without any recipient strain (●): it indicates the amount of transcription leakage in the presence of IPTG, with the *lacI*^{s} allele (around 5 units). After a 40 min conjugation period on filter, β-galactosidase activity of cell extracts was measured as a function of incubation time at 28°C.

The recombination assay, which is depicted in Figure 1B, was inspired from an earlier report (Birge and Low, 1974), except that other *lacZ* alleles were used. The donor strain contained a *lacZ* C-terminal deletion, and shared 2.7 kb homology within *lacZ* with the recipient strain, which contained a *lacZ* N-terminal deletion. When the Δ*lacZ* region from the donor enters the recipient strain, if it replicates and recombines into the 2.7 kb *lacZ* region with the recipient chromosome, an intact gene is restored. As a result, the β-galactosidase activity of the extracts will depend on both replication and recombination efficiencies.

Table I Quantifications of the kinetics of recombination and replication as shown Figure 1

	Recombination[a]		Replication[b]		Recomb./Replic.
	Ratio/ wt	Ratio/ pBR	Ratio/ wt	Ratio/ pBR	
wt	1.00		1.00		1.00
uvrD	1.94		0.93		2.09
Rep	0.58		0.60		0.98
recF	0.31		0.92		0.34
recB	0.10		0.82		0.12
recB recF	0.01		0.78		0.01
pBR	0.63	1.00	0.87	1.00	
UvrD++		0.55		0.79	0.70

[a]The slopes of the kinetics were measured between 15 and 60 min, except for strains *recB*, *recF*, and *recB recF*, where values between 0 and 120 min were taken. *R*-values were between 0.93 and 0.99. Each slope value was then divided by the slope value of the WT strain (column ratio/wt), or of the strain containing pBR322 (column ratio/pBR).
[b]Slopes were measured between 0 and 60 min. *R*-values ranged between 0.92 and 0.99.

Before testing helicase mutants, strains mutated in *recA*, *recB* and *recF* genes, three major players in homologous recombination, were monitored for recombination during 4 h after conjugation (Figure 2A). The *recA* mutant exhibited no recombination activity, as expected (Birge and Low, 1974). The *recB* mutant exhibited a strong defect in recombination (10% of wild-type (WT) activity, as measured by comparing slopes) (Table I) and the *recF* mutant a moderate defect (30% of WT). In the double *recB recF* mutant, recombination was completely abolished. These observations are also in agreement with previously reported data (Birge and Low, 1974; Lloyd *et al*, 1987b), and confirm that both RecF and RecB promote recombination in this assay. We concluded that the assay was reliable to examine recombination in real time.

In a similar way, *dnaEts* (*dnaE* encodes the α-subunit of DNA polymerase III) and *dnaBts* (*dnaB* encodes the replicative helicase) mutants were used to measure replication with our assay. β-Galactosidase activity was reduced compared to the WT strain (Delmas and Matic, manuscript in preparation). We then proceeded to analyse replication (Figure 2B) in the *rec* strains. Compared to the WT strain, replication was slightly affected in the *recB*, *recF* and *recB recF* mutants (80–90% of WT) (Table I). The difference of β-galactosidase activity between the *recB recF* strain, in which no recombination took place, and the WT strain, in which incoming DNA did recombine, could reflect the contribution of recombination events to total β-galactosidase activity. In this case, the contribution of recombination is low and does not mask the replication efficiency. In sharp contrast with the *recB recF* strain, almost no β-galactosidase activity was detected in the *recA* strain extracts. Rather than reflecting a replication defect, we suspect that this is due to efficient degradation of replicated DNA by RecBCD in the absence of RecA, as previously observed (Skarstad and Boye, 1993). Indeed, in the *recA recD* double mutant, which is devoid of RecBCD

exonuclease activity, β-galactosidase activity was partially restored. We concluded that the replication assay was a reliable means of detecting the fate of incoming DNA in the recipient strain, reflecting both its replication and its degradation, with little interference, if any, of recombination events.

UvrD directly prevents homologous recombination, and Rep promotes replication in vivo

Replication and recombination were then monitored in a *uvrD* mutant. We found that recombination was increased by a factor of two as compared to the WT strain (Figure 2C and Table I). In the replication assay, β-galactosidase activity in the *uvrD* strain was only slightly diminished (93% of WT), thus showing that the *uvrD* strain did not suffer any major replication defect. A strain overproducing UvrD was also tested (Figure 2C, UvrD++); replication was slightly affected (80% of WT) in this context, while recombination was reduced by a factor of two, as compared to the WT strain harbouring the control plasmid pBR322. Taken together, these data suggest that UvrD does not participate in DNA replication, but primarily and directly prevents recombination *in vivo*.

Replication and recombination were also analysed in a *rep* mutant. Replication was reduced (60% of WT; Figure 2F and Table I), suggesting that Rep is needed for replication of incoming DNA. Recombination was reduced as well (60% of WT; Figure 2E and Table I); this effect may simply be due to the decrease of replication.

As helicases are known to play various roles in a cell, it seemed important to test whether transcription and translation of the *lacZ* gene were affected in the *uvrD* and *rep* mutants and in the UvrD overproducing strain. Upon IPTG induction, *lacZ* expression was similar in WT, *uvrD*, *rep* and UvrD overproducing strains (not shown). We conclude that neither the helicase mutants nor the UvrD overproducing strain interfere with transcription/translation of *lacZ*.

The recA730 mutation severely affects viability of the rep uvrD recF mutant

Genetic data revealed that lethality of the *uvrD rep* double mutant is suppressed by mutations in *recF*, *recO* or *recR* genes (Petit and Ehrlich, 2002). The RecFOR proteins promote formation of RecA nucleofilaments on ssDNA gaps (Umezu *et al*, 1993; Morimatsu and Kowalczykowski, 2003). In *uvrD rep* cells, we suspected that the cause of toxicity may be unprocessed RecA nucleoprotein filaments themselves. In this scheme, the lethality of the *rep uvrD* mutant should be suppressed also by a *recA* mutation, but we have already shown that this was not the case (Petit and Ehrlich, 2002). The *recA* gene has a second role in *E. coli*, that is, induction of the SOS response, by stimulating cleavage of the LexA repressor. We therefore asked whether a strain in which the SOS response is constitutively expressed, due to inactivation of *lexA*, would allow *rep uvrD recA* cells to be viable. To test this, an experiment was conducted with the *rep*-encoding plasmid pAMrep, whose replication (based on pAM34; Gil and Bouche, 1991) relies on an IPTG-dependent promoter. The principle consists of constructing strains with the desired mutations in the presence of the plasmid providing Rep, to overcome the putative lethality of the *rep uvrD* combination.

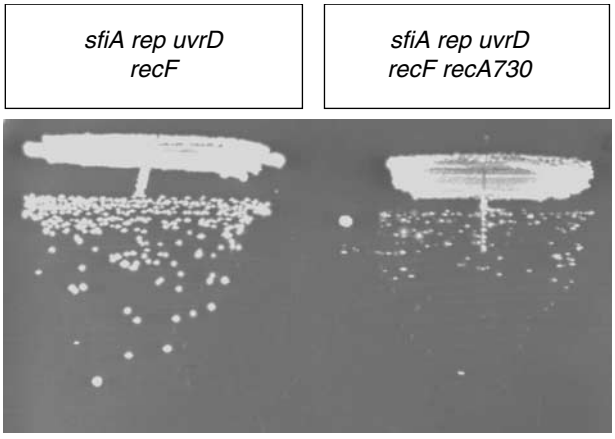

Figure 3 The *sfiA rep uvrD recF recA730* (MAC1159) grows poorly compared to its recA+ parent (MAC1168). Both strains were streaked on an LB plate and incubated for 48 h at 37°C.

The plasmid is then chased from the strain by growing cells in the absence of IPTG. If cells having segregated the plasmid continue to grow, the resulting mutant is viable. The results of segregation experiments are presented in Supplementary Figure 1S. It was not possible to recover plasmidless cells from the *lexA rep uvrD recA* pAMrep strain grown at 37°C (MAC1107), indicating that, even in an SOS constitutive background, the *rep uvrD recA* combination is lethal. The *rep recA* double mutant was reported to plate efficiently at 42°C and not at 30°C (Bredeche *et al*, 2001). The segregation experiment was therefore repeated at 42°C for the *rep recA lexA uvrD* and its RecA+ parent. Again, no plasmidless cell could be recovered at 42°C in this background. However, to our surprise, the *lexA rep uvrD* parent strain was viable at 42°C (see Discussion). Therefore, while removal of the RecFOR complex is beneficial in the *rep uvrD* strain, RecA is essential in this context. RecA filaments are formed with the help of either RecBCD or RecFOR. In *uvrD rep* mutants, the *recF, recO* and *recR* mutations might prevent the formation of RecA filaments at some places where they would be toxic, but not on RecBCD-generated structures that would require RecA for repair. This would explain the differential effect of *recF* and *recA* mutations on *uvrD rep* lethality.

To test the hypothesis that toxicity of RecFOR in *uvrD rep* mutant relates to RecA binding, we used the *recA730* allele that is a partial suppressor of *recF* (Wang *et al*, 1993). Suppression is due to the ability of RecA730 to compete with SSB, thereby bypassing a need for RecFOR to do so (Lavery and Kowalczykowski, 1992). We asked whether the *rep uvrD recF* strain (MAC1136) would be affected by addition of the *recA730* allele (strain MAC1146). This proved to be the case: the *rep uvrD recF recA730* mutant was still viable but it grew more slowly than the RecA+ isogenic strain (Figure 3), and also accumulated suppressors. We therefore concluded that the RecA nucleoprotein filament was a factor of toxicity in the *rep uvrD* background.

UvrD but not Rep inhibits RecA-mediated strand exchange in vitro

The above *in vivo* experiments led to the view that UvrD helicase, but not Rep helicase, plays a role directly connected with homologous recombination, and eventually with RecA

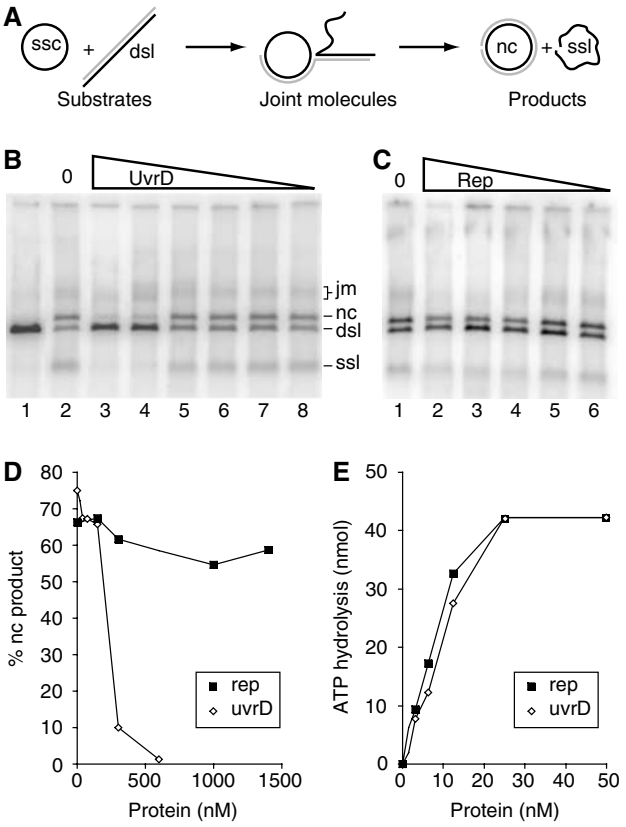

Figure 4 UvrD helicase but not Rep helicase inhibits strand exchange catalysed by RecA. (**A**) Scheme of DNA strand exchange reaction (ssc, single-stranded circular DNA; dsl, double-stranded linear DNA; jm, joint molecules; nc, nicked circular double-stranded DNA; ssl, single-stranded linear DNA). (**B**) After RecA nucleoprotein filament formation by preincubation of ssDNA with RecA and SSB proteins, various amounts of UvrD helicase were added simultaneously with ^{32}P end-labelled linear dsDNA, which initiates the strand exchange (lane 1: labelled dsl; lanes 2–7 correspond to 0, 600, 300, 150, 75, 37.5 and 18.75 nM UvrD, respectively). After incubation for 40 min at 37°C, reaction mixture was deproteinized and resolved onto a 0.8% agarose gel. (**C**) Same as in (B) except that Rep was used in place of UvrD (lanes 1–6 correspond to 0, 1400, 1000, 600, 300 and 150 nM Rep, respectively). (**D**) Quantification of the reactions shown in panels B and C. (**E**) Comparable ATPase activities of UvrD and Rep proteins. Reactions containing 1 mM ATP, saturating amount of synthetic oligo(dT)$_{55}$ (5.5 μM nucleotides) and increasing amount of proteins were incubated at 37°C for 15 min. The calculated amount of ATP hydrolysed linked to the oxidation of NADH was determined by measuring the OD at 340 nm.

itself. To test this hypothesis more directly, we compared the effects of UvrD and Rep helicases on an *in vitro* RecA-promoted DNA strand exchange reaction. In this assay, circular ΦX174 ssDNA was first incubated for 10 min with RecA and SSB proteins to form the nucleoprotein filament. The helicase and 5′ end-labelled *MfeI*-linearized ΦX174 dsDNA were then sequentially introduced (Figure 4A). In agreement with an earlier report (Morel *et al*, 1993), we found that addition of increasing amounts of UvrD to the reaction strongly inhibited the strand transfer. Almost no nicked circular duplex was detected at a UvrD concentration of 600 nM (Figure 4B and D). In contrast, Rep protein did not affect strand exchange reaction even at concentrations up to 1.4 μM (Figure 4C and D).

We observed an abrupt change between 150 and 300 nM UvrD helicase concentrations. This effect was not due to ATP depletion because increasing three times the ATP regenerating system did not change the shape of the curve (data not shown).

Rep and UvrD were both similarly active in this assay, as revealed by their specific ATPase activities (Figure 4E). Thus, the fact that only UvrD inhibits the strand exchange reaction reflects an intrinsic property of this helicase.

RecA–ssDNA nucleoprotein filament is efficiently dismantled by UvrD but not by Rep

The yeast Srs2 helicase performs its antirecombinase action by disrupting Rad51 nucleoprotein filaments (Krejci *et al*, 2003; Veaute *et al*, 2003). We considered the possibility that similarly to Srs2, UvrD inhibits RecA recombinase function by releasing this protein from the presynaptic filament. We also hypothesized that Rep does not disrupt the RecA nucleoprotein filament because of the absence of any inhibitory effect on strand exchange. Electron microscopy was used to characterize the action of UvrD and Rep on the RecA presynaptic filament. RecA was first incubated for 5 min with ΦX174 ssDNA in the presence of SSB protein, in order to assemble nucleoprotein filaments (Figure 5A). The reaction was then diluted five times before introduction of the helicase. Subsequent to the addition of UvrD, we observed the appearance of ssDNA molecules covered by SSB (such

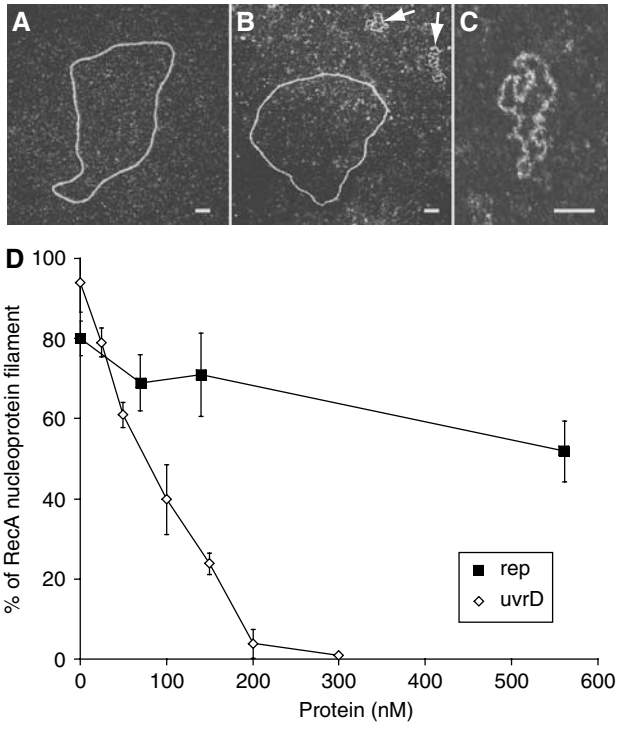

Figure 5 UvrD helicase, but not Rep helicase, efficiently disrupts RecA–ssDNA nucleoprotein filament. (**A**) RecA–ssDNA nucleoprotein filaments. (**B**) Preformed RecA–ssDNA complexes were incubated for 15 min with UvrD. The arrows point to the ssDNA covered with SSB. (**C**) Blow-up of ssDNA covered with SSB. (**D**) The percentage of disrupted RecA presynaptic filament was determined for different amounts of both helicases. For each concentration, mean values and standard deviations were obtained from two series of 300 molecules counted. Scale bars, 50 nm.

molecules appear as small bushes, shown with a white arrow, as opposed to the well-spread RecA nucleofilaments), resulting from disruption of the RecA filament (Figure 5B–D). Dilution did not perturb the RecA filament (Figure 5D). Progressive loss of RecA filaments, coupled with concomitant formation of SSB–ssDNA complexes, was observed as a function of the amount of UvrD introduced into the reaction. At 200 nM UvrD, more than 95% of the observed DNA molecules were covered by SSB. This amount of UvrD is about 10 times lower than that of RecA, indicating that UvrD acts catalytically. *In vivo*, the UvrD concentration is predicted to range from 1.4 to 4 μM (1000–3000 molecules/cell) (George *et al*, 1994). Therefore, the range of UvrD concentrations active *in vitro* is relevant to the physiological conditions. The dilution step, after RecA filament assembly, was needed to see UvrD activity: when the helicase was added to the undiluted reaction, the same range of UvrD concentrations had no apparent effect. Only with a higher concentration of 1 μM UvrD could we detect partial RecA removal. We believe that, in the absence of dilution, a rapid reassociation of RecA with the ssDNA precludes SSB binding to ssDNA.

Unlike the effect of UvrD, introduction of the Rep helicase to the reaction did not severely perturb the RecA presynaptic filaments. Large amounts of Rep were needed to trigger a small effect; less than 30% of the nucleoprotein filaments were dislocated after incubation with 560 nM Rep (Figure 5D). *In vivo*, the Rep concentration is predicted to be around 70 nM (50 molecules/cell) (Scott and Kornberg, 1978). This suggests that the effect of Rep *in vitro* does not reflect a physiological function of this helicase.

Taken together, the data from strand exchange experiments and from electron microscopic analyses indicate that the UvrD but not the Rep helicase acts as an antirecombinase by disrupting RecA nucleoprotein filaments.

Discussion

We found that UvrD, but not Rep, removes RecA from DNA. Keeping this in mind, some of the *uvrD* phenotypes may be interpreted as follows: the hyper-recombination nature of the *uvrD* mutant would result from a shift towards a higher level of recombination products, because RecA nucleoprotein filaments are not removed by UvrD and the essential activity of UvrD in the *rep* mutant would be due to its role as 'RecA remover' at a critical place or moment in the cell.

UvrD removes RecA from DNA

We report here that UvrD helicase disrupts RecA nucleofilament *in vitro*. Morel *et al* (1993) previously showed that UvrD could block the progression of a RecA-mediated strand exchange reaction, and proposed that the role of UvrD was to unwind the dsDNA and thereby reverse the reaction. Our electron microscopic studies suggest rather that strand exchange is stopped because RecA is wiped away from DNA. This model provides a simple explanation for many earlier observations, showing that *uvrD* mutants are hyper-recombinogenic. Until the impact of the *uvrD* mutation on DNA replication was studied, one could not exclude the possibility that the absence of UvrD impedes DNA replication, leading to frequent recombinogenic structures, as observed for various replication mutants (Bierne *et al*, 1997b; Flores *et al*, 2001). Our *in vivo* system, based on DNA conjugation, brings evidence that replication is almost unperturbed in the *uvrD* mutant, while recombination is increased. We can therefore propose a new role for UvrD *in vivo*, which is to remove RecA nucleoprotein filaments. UvrD had already been found to remove proteins from DNA, but always in a situation coupled with DNA unwinding: in the context of UvrABC-dependent UV repair, after the incision step mediated by UvrABC, the UvrD helicase was found to remove both the 12-mer containing the lesion and the UvrC protein (Orren *et al*, 1992). UvrD also removes the Tus protein bound to its *ter* site *in vitro* (Hiasa and Marians, 1992), the topoisomerase IV from its cleaved DNA–substrate intermediate (Howard *et al*, 1994) and LacI from its DNA operator site (Yancey-Wrona and Matson, 1992). The present work provides evidence that UvrD is also active through its translocase activity on ssDNA to remove RecA nucleoprotein filaments. More generally, DNA and RNA helicases may not limit themselves to a role in DNA or RNA unwinding, but bring also an activity of protein removers, while tracking on their RNA or DNA substrate (Jankowsky *et al*, 2001; Fairman *et al*, 2004). One may also interpret in this light the surprising recent observation that UvrD has a strand-switching activity, which results in reannealing the portion of dsDNA it has unwound (Dessinges *et al*, 2004). During such a strand switching *in vivo*, all RecA molecules bound, for example to a stalled replication fork, could be displaced, so that the final balance of the operation is non-nil (see below).

UvrD behaves like Srs2

Our results with UvrD generalize to prokaryotes what has been reported recently concerning the Srs2 helicase of *S. cerevisiae*: These two helicases remove RecA or Rad51 nucleoprotein filament, respectively. Furthermore, Srs2 helicase was shown to disrupt RecA nucleoprotein filaments (Krejci *et al*, 2003; Veaute *et al*, 2003). We found that UvrD can also dismantle presynaptic filaments of Rad51 (data not shown). UvrD and Srs2 helicases are therefore efficient at removing nucleoprotein filaments across species. Srs2 was found to interact physically with Rad51 (Krejci *et al*, 2003). However, the precise way these helicases recognize the filament structure remains to be determined.

Rep acts at the replication stage

We found that Rep cannot replace UvrD in the RecA displacement activity *in vitro*. *In vivo*, the behaviour of the *rep* mutant is completely different from that of the *uvrD* mutant. Replication of transferred DNA during conjugation is reduced in the *rep* mutant (Figure 2F). Chromosome replication forks are reportedly twice slower in a *rep* mutant (Lane and Denhardt, 1975; Colasanti and Denhardt, 1987). Based on the observation that Rep efficiently unwinds LacI-bound dsDNA *in vitro* (Yancey-Wrona and Matson, 1992), it is generally assumed that Rep acts ahead of the replicative DNA polymerase to remove protein road-blocks. However, during conjugative replication, the DNA ahead of the DNA polymerase is single stranded rather than double stranded, and there is no replication fork. It seems therefore difficult to assume that Rep acts at the same level 'ahead of the fork'. Alternatively, the defect of the *rep* mutant could be related to a default of DnaB loading. Replication during conjugation depends on DnaB (Willetts and Wilkins, 1984), whose role is probably to bring the DnaG primase to the DNA. The proteins

needed to load DnaB onto the DNA in this particular case are not known, but Rep, together with PriC, has been proposed as an alternate pathway for DnaB loading, in the absence of PriA (Sandler, 2000). A role of Rep during Okazaki fragment synthesis cannot be excluded, but so far our attempts to detect it have failed (data not shown). Moreover, strains defective for lagging strand synthesis are reportedly hyper-recombinogenic (Flores *et al*, 2001), which is not the case of the *rep* mutant. Clearly, understanding the role of Rep *in vivo* remains a challenge for the future.

What is the essential role of UvrD in the rep mutant?

Based on the above-mentioned observations, it seems unlikely that Rep and UvrD substitute for each other in an essential process *in vivo*, which leads to the death of the *rep uvrD* mutant. On the contrary, as Rep acts in replication, we suggest that, in its absence, structures are generated that require UvrD for processing. Interestingly, it was recently reported that UvrD is needed for replication fork reversal in two different DNA polymerase III mutants (Flores *et al*, 2004). This points to the capacity for the UvrD helicase to be active at a fork. Fork reversal designates the capacity of a blocked fork to unwind its two nascent strands and anneal them together (Higgins *et al*, 1976). Such a process could permit replication restart. It was proposed to be frequent in the *rep* mutant (Seigneur *et al*, 1998). The direct study of fork reversal in the *rep uvrD* mutant is prevented by its inviability. However, if UvrD also acts in fork reversal in the *rep* mutant, and considering that in this mutant UvrD is important for the removal of RecA nucleoprotein filaments, the following scenario can be envisaged: (1) The replication fork is sometimes pausing due to the absence of Rep, and the replication apparatus collapses. (2) A particular DNA structure, unknown at present, is generated at the fork, containing ssDNA, which we will call 'forked DNA'. (3) A RecA nucleoprotein filament is loaded by RecFOR on the single-stranded part of the 'forked DNA' and this complex cannot be further processed by recombination. (4) UvrD is essential at this moment to remove RecA, and permit either fork reversal or the loading of a new replication apparatus. The toxicity conferred by the *recA730* mutation to the *rep uvrD recF* cells is explained by the ability of RecA730 protein to bind ssDNA at the 'forked DNA' in the absence of RecFOR. This scenario assumes that RecF, RecO and RecR can bind to ssDNA at a fork, which is different from a '*bona fide*' single-stranded gap. In support of this view, it was observed that RecFOR protects newly replicated DNA extremities after UV irradiation (Courcelle *et al*, 1997; Chow and Courcelle, 2004).

A recF, but not a recA mutation suppresses rep uvrD lethality

The above-mentioned scenario does not explain why a *recA* mutation does not suppress *rep uvrD* lethality. If RecA had only a toxic effect in the *rep uvrD* background, its complete removal should save the strain. It could be that RecA is important for RecBCD-dependent recombination in the *rep* strain, in a situation unrelated to fork pausing. A simple possibility is that, due to the absence of UvrD, which plays a role in mismatch and UV repair, nicks are more frequent in the chromosome. Bradshaw and Kuzminov (2003) reported that replication 'across' such nicks leads to fork collapse, formation of double-stranded ends and subsequent repair by RecBCD and RecA. Although the *uvrD recA* double mutant is viable, it is possible that the additive *rep* defect provokes a cell crisis. Finally, a whole set of observations leads to the conclusion that growth at 42°C is beneficial to some helicase mutant derivatives: the *rep recA* strain, the *rep uvrD* strain (the observation made in the *sfiA11 lexA rep uvrD* was reproduced in the *sfiA11 rep uvrD* and *rep uvrD* strains: all strains, which were not viable at 37°C, could be constructed at 42°C, data not shown) and also the *lexA uvrD recA* strain (M-A Petit, unpublished observation). These observations are paradoxical given the fact that more replication forks are present at 42°C compared to 37°C, so that more replication pausing and more problems are expected. This is clearly an exciting field of investigations for the future.

In conclusion, our data show that the lethality in *rep uvrD* mutants is not a result of the overlapping functions of both helicases. UvrD, but not Rep, plays a role of RecA nucleoprotein filament remover in *E. coli*. The UvrD helicase proves therefore to play a new role, unrelated to DNA melting, *in vivo*. While studying the precise tracking mechanism of the PcrA helicase, Wigley and his collaborators had envisaged that an important part of the energy consumed while translocating may be used for some other purpose, like protein removal (Soultanas and Wigley, 2001). By extending the results obtained recently for the *S. cerevisiae* Srs2 helicase, which disrupts Rad51 nucleoprotein filaments (Krejci *et al*, 2003; Veaute *et al*, 2003), our data suggest effectively that DNA helicases play important roles as protein removers *in vivo*.

Materials and methods

Strains and plasmids

Strains and plasmids used are listed in Table II. Several mutations were constructed using a technique relying on the Lambda recombination genes (Datsenko and Wanner, 2000): A new *uvrD* null allele was needed for this study, because of the instability of the transposon insertion alleles, such as *uvrD*::Tn*5*. The phleomycin resistance gene of pUB110, fused to the *B. subtilis sacB* promoter, was amplified by PCR from the pUC-phleo plasmid (a gift from E Dervyn), using two long primers containing also 50-nt-long regions homologous to the start and end regions of the *uvrD* gene. This fragment was then used to replace the WT *uvrD* gene, between positions −80 and +2600 relative to the +1 start codon of *uvrD*. For the same reason of transposon instability, a *lexA*::Km^R allele was also constructed, in which the *lexA* gene, between positions +1 and +608 relative to the +1 start codon of *lexA*, was replaced by the Km^R gene of pKD4 (Datsenko and Wanner, 2000). Two precise deletion alleles of *lacZ*, used for the conjugation assays, *lacZΔP* and *lacZΔT*, were also constructed. In *lacZΔP*, the region between −138 and +10, relative to the +1 start codon of *lacZ*, was replaced by the Cm^R cassette of pKD3 (Datsenko and Wanner, 2000), and in *lacZΔT*, the same cassette was placed between +2798 and +3051 of *lacZ*. The *lacZΔP* deletion deleted also the C-terminal part of LacI. These alleles were transduced into the relevant strains by P1 transduction, and the CmR cassette was removed by site-specific recombination between FRT sites flanking the gene, using pCP20, as described (Datsenko and Wanner, 2000). To verify the introduction of the *recA730* allele in strain MAC1146, two oligonucleotides ending at the position of the differing nucleotide (a G in WT *recA*, and an A in *recA730*, at +115 relative to the start codon) and specific for the WT *recA* or the *recA730* allele were used in a PCR with a compatible downstream oligonucleotide.

Conjugational crosses and β-galactosidase assays

Overnight cultures were diluted 50-fold in LB for donor cells, or LB supplemented with IPTG (100 μM) for recipient cells, and grown to

Table II *E. coli* strains and plasmids

Strain	Relevant genotype or phenotype	Source/construction
MG1655	Wild type	Radman laboratory stock
Nec220	As MG1655 but Nal^R	Spontaneous Nal^R clone
JJC40	AB1157 *hsdR*	B Michel laboratory stock
Hfr3000	oriT near 0 min	Radman laboratory stock
CGSC6378	*lacI*^{s}	Willson *et al* (1964)
JJC451[a]	*recF400*::Tn*5*	B Michel
JJC760[a]	*rep*::Cm^R (pGB2Tsrep)	B Michel
JJC1397[a]	*sfiA11*	B Michel
JM103recA730	*recA730 srl*::Tn*10 sfiA*::Tn*5*	R Fuchs
GY5902	Δ*recA306 srl*::Tn*10* (miniF *recA*)	S Sommer
N2101	*recB268*::Tn*10*	Lloyd *et al* (1987a)
DB1318	*recA938*::Tn*9-200*	Wertman *et al* (1986)
CGSC7429	*recD1901*::Tn*10*	Genetic stock centre
FR705	*proC*::Tn*5*	Radman laboratory strain
Nec235	*proC*::Tn*5 lacI*^{s}	P1 FR705*CGSC6378
Nec221[b]	*lacZΔP*::Cm^R	Red-Gam-mediated gene replacement
Nec222[b]	*lacZΔT*::Cm^R	Red-Gam-mediated gene replacement
MAC833[a]	*uvrD*::$Pheo^R$	Red-Gam-mediated gene replacement
MAC810[a]	*sfiA11 lexA*::Kan^R	Red-Gam-mediated gene replacement
MAC448[a]	*rep*::Cm^R	P1 JJC760*JJC40
Nec223[b]	*lacZΔP*	Excision of the CmR cassette from Nec221
Nec227[b]	*lacZΔP recF400*::Tn*5*	P1 JJC451*Nec223
MAC1058[b]	*lacZΔP uvrD*::$Pheo^R$	P1 MAC833*Nec223
MAC1065[b]	*lacZΔP rep*::Cm^R	P1 JJC760*Nec223
MAC1068[b]	*lacZΔP* (pBR322)	Plasmid transformation
MAC1071[b]	*lacZΔP* (pGT26)	Plasmid transformation
Nec228[b]	*lacZΔP recB268*::Tn*10*	P1 N2101*Nec223
Nec229[b]	*lacZΔP recA938*::Tn*9-200*	P1 DB1318*Nec223
Nec231[b]	*lacZΔP recD1901*::Tn*10*	P1 CGSC7429*Nec223
Nec230[b]	*lacZΔP recA938*::Tn*9-200 recD*::Tn*10*	P1 DB1318*Nec231
Nec232[b]	*lacZΔP recB268*::Tn*10 recF400*::Tn*5*	P1 N2101*Nec227
Nec224	Hfr3000 *lacZΔT*::Cm^R	P1 Nec221*Hfr3000
Nec225	Hfr3000 *lacZΔP*::Cm^R	P1 Nec222*Hfr3000
Nec226	Hfr3000 *proC*::Tn*5 lacI*^{s}	P1 Nec235*Hfr3000
MAC680[a]	*sfiA11 rep*::Cm^R	P1 JJC760*JJC1397
MAC1059[a]	*sfiA11 rep*::Cm^R (pAMrep)	Plasmid transformation
MAC1077[a]	*sfiA11 rep*::Cm^R *uvrD*::$Pheo^R$ (pAMrep)	P1 MAC833*MAC1059
MAC1089[a]	*sfiA11 rep*::Cm^R *uvrD*::$Pheo^R$ *lexA*::Kan^R (pAMrep)	P1 MAC 810*MAC1077
MAC1107[a]	*sfiA11 lexA*::Kan^R *rep*::Cm^R *uvrD*::$Pheo^R$ Δ*recA306 srl*::Tn*10* (pAMrep)	P1 GY5902*MAC1089
MAC1136[a]	*sfiA11 rep*::Cm^R *uvrD*::$Pheo^R$ *recF400*::Tn*5* (pAMrep)	P1 JJC451*MAC1077
MAC1146[a]	*sfiA11 rep*::Cm^R *uvrD*::$Pheo^R$ *recF400*::Tn*5 recA730 srl*::Tn*10* (pAMrep)	P1 JM103recA730*MAC1136
MAC1168[a]	*sfiA11 rep*::Cm^R *uvrD*::$Pheo^R$ *recF400*::Tn*5*	MAC1136 cured of pAMrep
MAC1159[a]	*sfiA11 rep*::Cm^R *uvrD*::$Pheo^R$ *recF400*::Tn*5 recA730 srl*::Tn*10*	MAC1146 cured of pAMrep
MAC1165[a]	*sfiA11 rep*::Cm^R *uvrD*::$Pheo^R$	MAC1077 cured of pAMrep at 42°C
Plasmids		
pGT26	pBR328 derivative encoding *uvrD*	Taucher-Scholz and Hoffmann-Berling (1983)
PAMrep	pAM34 derivative encoding *rep*	B Michel

[a]JJC40 background (AB1157 derivative).
[b]Nec220 background (MG1655 derivative).

an OD_{530} of 0.9 (about 10^8 cells/ml). When the recipient strain contained a plasmid, ampicillin (100 μg/ml) was present in the medium. In all, 2 ml of donor and 2 ml of recipient cells were then mixed, and deposited on a nitrocellulose filter. Conjugation was allowed to proceed by placing the filter on a prewarmed LB plate containing 100 μM IPTG, for 40 min at 37°C. After this 40 min period, conjugation was interrupted and this corresponded to time zero of the experiment. Cells were resuspended in 2 ml of a chemically defined medium M9 lacking a carbon source, to reduce cell growth to its minimum (M9 was supplemented with 30 μg/ml thiamine, 0.002% uracil, 3 mM $MgSO_4$ and 0.1% casaminoacids), and separated by vortexing. Nalidixic acid (40 μg/ml) was added to kill donor cells, as well as IPTG (100 μM), and the cultures were maintained at 37°C under agitation for the time course of the experiment. Samples of 0.1 ml (replication assay) or 0.2 ml (recombination assay) were withdrawn at appropriate time points, and treated for the β-galactosidase assay as described (Miller, 1972). To test whether *lacZ* transcription and/or translation were affected in helicase mutant and helicase overproducing strains, a time course of β-galactosidase activity after IPTG induction was performed in parallel in the JJC40 strain, the MAC448 *rep* derivative and MAC833 *uvrD* derivative. Induction of *lacZ* expression was equally efficient in all three strains (not shown).

Protein and DNA reagents

UvrD and Rep were gifts from Era Cassuto and Ken Marians, respectively. RecA was purchased from Roche and SSB protein was from Amersham Biosciences. ΦX174 viral (+) ssDNA and ΦX174 RF1 dsDNA were purchased from Biolabs.

DNA strand exchange reaction

ΦX174 ssDNA (4.5 μM nucleotides) was incubated in buffer A (30 mM Tris–HCl (pH 7.6), 9 mM $MgCl_2$, 1.8 mM DTT, 1.1 mM ATP, 7.2 mM phosphocreatine, 9 U/ml phosphocreatine kinase) for 3 min at 37°C before the addition of 1.5 μM RecA protein and 0.26 μM SSB. The reaction was kept at 37°C for 10 min. Strand exchange was started by addition of 5′ ^{32}P end-labelled *Mfe*I-linearized ΦX174 dsDNA (4.5 μM nucleotides) and variable amounts of helicases.

After 40 min incubation, reaction mixture was deproteinized and analysed by electrophoresis in 0.8% agarose gel in TAE buffer. Gels were dried and quantified by ImageQuant software. Percentage of nicked circular products was calculated as follows:

$$(2\times \text{AU of nicked circular band})/(\text{AU of linear band} + 2\times \text{AU of nicked circular band})$$

The signal of the nicked circular band was multiplied by 2, because only one labelled strand was transferred to the single-stranded circular DNA. AU stands for arbitrary units given by the ImageQuant software.

ATPase assay

ATPase activity was assayed by linking ATP hydrolysis to NADH oxidation, as described previously (Pullman *et al*, 1960). The reactions were carried out at 37°C for 15 min in 50 mM Tris–HCl (pH 7.6), 100 mM NaCl, 7 mM $MgCl_2$, 80 μg/ml BSA in the presence of 1 mM ATP and saturating amount of synthetic oligo$(dT)_{55}$ (5.5 μM nucleotides). The concentrations of proteins UvrD and Rep used in this assays are indicated in the figure legend.

Electron microscopy

RecA filaments on ssDNA were formed by incubation of 2.46 μM RecA and 0.41 μM SSB protein in DNA strand exchange buffer with 4.9 μM (nucleotides) ΦX174 viral (+) single strand for 5 min at 37°C. Reactions mixtures were then diluted five times in TNM (10 mM Tris–HCl (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$) or buffer A before subsequent addition of variable amounts of helicase for 15 min. After an additional dilution (four times) in TNM, the products of the reaction were analysed by electron microscopy as described previously (Beloin *et al*, 2003).

Supplementary data

Supplementary data is available at *The EMBO Journal* online.

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