Replication Fork Reversal after Replication–Transcription Collision

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Abstract

Replication fork arrest is a recognized source of genetic instability, and transcription is one of the most prominent causes of replication impediment. We analyze here the requirement for recombination proteins in Escherichia coli when replication–transcription head-on collisions are induced at a specific site by the inversion of a highly expressed ribosomal operon (rrn). RecBC is the only recombination protein required for cell viability under these conditions of increased replication–transcription collisions. In its absence, fork breakage occurs at the site of collision, and the resulting linear DNA is not repaired and is slowly degraded by the RecJ exonuclease. Lethal fork breakage is also observed in cells that lack RecA and RecD, i.e. when both homologous recombination and the potent exonuclease V activity of the RecBCD complex are inactivated, with a slow degradation of the resulting linear DNA by the combined action of the RecBC helicase and the RecJ exonuclease. The sizes of the major linear fragments indicate that DNA degradation is slowed down by the encounter with another rrn operon. The amount of linear DNA decreases nearly two-fold when the Holliday junction resolvasen RuvABC is inactivated in recB, as well as in recA recD mutants, indicating that part of the linear DNA is formed by resolution of a Holliday junction. Our results suggest that replication fork reversal occurs after replication–transcription head-on collision, and we propose that it promotes the action of the accessory replicative helicases that dislodge the obstacle.

Introduction

Replication arrest is a recognized source of genetic instability in all organisms. Proteins that protect, process, and restart arrested replication forks have been identified, and in eukaryotes their action is coordinated with the induction of a check-point response to prevent cell cycle progression until replication resumes [1,2,3,4]. Model organisms have been used to amplify specific causes of replication arrest in a controlled way, revealing the existence of dedicated pathways of replication resumption. In bacteria, it has been shown that in spite of the existence of several well-characterized replication-restart machineries capable of reloading a replisome at a replication fork, which depend on the nature of the obstruction, most often arrested replication forks do not simply restart [5]. They are first targeted by various enzymes including accessory replicative helicases and recombination proteins [6,7,8]. It thus appears that different causes of replication arrest trigger different responses, and that arrested replication forks are channeled to various pathways depending on the original cause of arrest.

One of the first replication impediments recognized as important is the one created by transcription [9,10,11,12,13]. Enzymes that facilitate replication across highly transcribed regions have been identified in yeast [14]. In bacteria, replication, transcription and translation occur concomitantly, and replication progresses more than 10 times faster than transcription. Consequently, replication-transcription collisions are predicted to occur quite frequently. Head-on collisions between replication and transcription are more dramatic than co-directional collisions [9,15,16], nevertheless, under standard growth conditions replication arrests in highly transcribed regions are frequent enough to turn them into detectable hotspots of replication restart [17].

In Escherichia coli, as in other organisms, recombination proteins have been shown to facilitate replication progression under various conditions of replication impairment [6,18]. In several replication mutants, recombination proteins play a specific role by participating in a reaction named replication fork reversal (RFR) [5,6,19,20,21]. During RFR, the newly synthesized strands are unwound from the daughter duplexes and base pair to form a Holliday junction (HJ) adjacent to a DNA double-stranded (dsDNA) end. This dsDNA end, made by the annealing of the leading and lagging strand ends, is used to reset a functional replication fork either by RecBCD- RecA- RuvABC- catalyzed homologous recombination (Figure 1A, pathway C), or by RecBCD- catalyzed DNA degradation (Figure 1A, pathway D). RecBC is essential for the viability of replication mutants that undergo RFR, because in its absence the dsDNA end is not efficiently processed, and resolution by RuvABC of the HJ produced by fork reversal results in the formation of a lethal, one-

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Author Summary

Genomes are duplicated prior to cell division by DNA replication, and in all organisms replication impairment leads to chromosome instability. In bacteria, replication and transcription take place simultaneously, and in eukaryotes house-keeping genes are expressed during the S-phase; consequently, transcription is susceptible to impair replication progression. Here, we increase head-on replication–transcription collisions on the bacterial chromosome by inversion of a ribosomal operon (rrn). We show that only one recombination protein is required for growth when the rrn genes are highly expressed: the RecBCD complex, an exonuclease/recombinase that promotes degradation and RecA-dependent homologous recombination of linear DNA. In the absence of RecBCD, we observe linear DNA that ends in the collision region. This linear DNA is composed of only the origin-proximal region of the inverted rrn operon, indicating that it results from fork breakage. It is partly RuvABC-dependent (i.e., produced by the E. coli Holliday junction resolvase), indicating that blocked forks are reversed. The linear DNA ends up at the inverted rrn locus only if the RecJ exonuclease is inactivated; otherwise it is degraded, with major products ending in other upstream rrn operons, indicating that DNA degradation is slowed down by ribosomal operon sequences.

A related reaction was later proposed to promote replication restart after blockage by a RNA polymerase that is itself arrested by a UV lesion [26]. Based on measures of cell survival after UV irradiation in various mutants, it was concluded that replication forks arrested by RNA polymerase in UV irradiated cells were reversed by RecG, an enzyme that binds multiple branched structures in vivo and in vitro [27]. In contrast with replication mutants, reversed forks in UV-irradiated cells were proposed to be targeted primarily by HJ-binding enzymes, and not by RecBCD: either RecG would convert them back to fork structures and thereby promote restart, or RuvABC would resolve them causing fork breakage. However, this model was later challenged, when direct measures of DNA synthesis showed that the inactivation of recG does not prevent replication restart in UV irradiated cells [28] and even promotes it [29,30]. Furthermore, quantification of UV-induced chromosome fragmentation in recBC mutants showed that it completely depends on RuvABC but is hardly influenced by the RecG status of the cell [31].

Interestingly, RNA polymerase is now recognized as the main replication obstacle in the rep mutant, where RFR was first described [8,32]. In E. coli, three accessory replicative helicases act at forks blocked by replication-transcription collisions: Rep, UvrD and DinG [8,33]. Rep is the most critical of the three helicases since it is the only one required for normal replication. In rep mutants chromosome replication is twice slower than in wild-type cells [34], probably owing to frequent replication arrest since rep mutants undergo RFR and need replication restart proteins for growth [19]. Furthermore, Rep is driven to arrested replication forks by a direct interaction with the replicative helicase DnaB [35]. UvrD can substitute for Rep in its absence, since the uvrD single mutant has no replication defect whereas cells that lack both Rep and UvrD have a very low viability [32,36]. DinG acts as a second back-up; its inactivation is not deleterious in rep or uvrD single mutants but prevents the residual growth of rep uvrD double
mutants, as well as the growth of rep wvd mutants in which viability is restored by a suppressor mutation that reduces replication arrest or limits its deleterious consequences (recE, rpoB, rpoC mutations, [8,32]).

In order to better characterize the action of enzymes recruited to replication-transcription collision sites, we constructed strains in which the inversion of a specific ribosomal operon (rrn) creates a strong, locally defined replication obstacle under rapid growth conditions, owing to the high level of rrn operon expression (Inv strains, Figure 1B; [8]). This particular experimental setup allowed us to directly demonstrate that in vivo Rep, UvrD and DinG helicases indeed act at sites of replication-transcription collisions. Furthermore, in Inv strains the presence of any combination of two of these three accessory replicative helicases is required for growth in rich medium, i.e. under high collision conditions, suggesting that two of these helicases act together [8]. In contrast, the transcription-coupled repair factor Mfd does not seem to play a role in the viability of Inv mutants (Table S1, Ref. in Text S1), although this helicase dislocates transcription complexes blocked in vivo by a DNA lesion and in vitro by various obstacles, including replication forks [37,38,39].

In the present work, we used strains carrying one or two inverted rrn operons (Inv mutants) to address the question of the role of recombination proteins following replication-transcription collisions. First, we tested the effects of several recombination mutations and combination of mutations on the viability of Inv mutants. We previously reported that Inv strains are not affected by the absence of RecF or RecA, essential for the repair of single-stranded DNA gaps [8]; we identify here the main DSB repair complex RecBC as the only recombination function essential for viability under conditions of high replication-transcription collisions. Second, we looked for the occurrence of DSBs in the region of replication-transcription collisions by direct analysis of chromosomes of different mutants; we actually observed chromosome breakage under conditions of hyper-collisions. Third, we characterized these chromosome breaks and show that only the origin-proximal DNA double-strand end of the replication-transcription collision site can be detected, which is indicative of fork breakage. We also show that linear DNA ends that result from fork breakage are slowly degraded even in the absence of Exo V, the major dsDNA exonuclease; we show that this DNA degradation is mainly RecJ-dependent and is slowed down upon the encounter of other rrn loci on the chromosome. Finally, we addressed the question of the enzyme(s) involved in the fork breakage reaction by inactivating candidate genes. Only recAB inactivation was found to affect the level of fork breakage, indicating a role for the RuvAB complex. Altogether, these results lead us to propose a model for the processing of replication forks arrested by the encounter of an oppositely-oriented highly-transcribed locus.

Results

Inv mutants require RecBC for growth on rich medium

In order to test the role of recombination proteins upon replication-transcription collisions we used the InvA and InvBE strains, which carry a 18 kb inversion encompassing rrnD, and a 138 kb inversion containing rrnB and rrnE, respectively (Figure 1B). rrn mutations increase the level of replication-transcription collisions particularly in rich medium (Luria Broth, LB), i.e. under conditions of high rrn expression and high replication fork density [8]. Recombinational repair of DNA double-stranded breaks (DSBs) requires (i) RecBC, the pre-synaptic protein that loads RecA at dsDNA ends, (ii) RecA, which promotes strand invasion and homology search, and (iii) RuvAB and RuvG (or RecG) which resolve Holliday junctions formed by strand exchange [22]. Inverted rrn genes do not render homologous recombination essential, as RecA is not required for the growth of Inv strains on LB (Figure 2; [8]). Similarly, we observed that the inactivation of RuvABC and/or RecG, which catalyze the final step of homologous recombination as two redundant pathways of Holliday resolution, did not affect the growth of Inv mutants on LB (Inv rec, Inv recG and Inv recD mutants, Figure 2; the 5-fold reduction of viability of the inv recG combination of mutations in minimal medium was similarly observed in non-inverted strains (data not shown), and the slightly higher reduction of viability in LB might result from an increased need for the resolution of homologous recombination intermediates). However, a recB null mutation, which by inactivating the RecBCD complex prevents both the recombinational repair and the degradation of dsDNA ends, strongly decreased the plating efficiency of Inv mutants on LB medium (Figure 2). This observation suggests that dsDNA ends, which need to be acted upon by RecBCD, are formed during replication of the inverted chromosome region. The recD mutation, which leaves intact the homologous recombination activity of the RecBC complex but inactivates the efficient dsDNA degradation function of RecBCD, exonuclease V, did not prevent growth of Inv mutants on LB medium, suggesting that in the absence of exo V the recombination function of RecBC (helicase and RecA loading activities) is sufficient for viability.

Although InvBE carries a large inversion, the LB sensitivity of Inv recB mutants is due to the inversion of the only highly expressed of the two inverted operons, rrnE, since the precise deletion of this operon restored 100% colony formation on LB compared to MM (InvBE ΔrrnE recB mutant, Figure 2). It should be noted that the inverted region in the InvBE ΔrrnE recB mutant still carries an inverted rrn locus, rrnB. However, rrnB is expressed here at the same level in LB and in MM (minimal medium), owing to a deletion of the promoter enhancer region [8]. The full viability of the InvBE ΔrrnE recB mutant shows that RecBC is only needed when the inverted rrn operon is highly expressed. In addition, a RNA polymerase mutation that decreases rrn operon expression in LB [8], strongly increased the viability of the InvBE recB mutant (InvBE rpoCA215–220 recB mutant, Figure 2). Finally, rrn genes are prone to the formation of R-loops [40]; however, the requirement for RecBC is not linked to R-loop formation since over-expression of RNaseH, which destroys R-loops, did not restore viability (InvBE recB pR; Figure 2). Altogether, these results suggest that the requirement for RecBC is caused by collisions between replication forks and RNA polymerases transcribing the inverted rrn operons.

The LB sensitivity of Inv recB mutants, together with the lack of LB sensitivity of Inv recD, and Inv recG mutants, suggest that a dsDNA end is formed upon replication collisions with the inverted rrn without actual DNA breakage. These results can be accounted for by the RFR model (Figure 1A). This model predicts that inactivation of both recombination (by a recA mutation) and RecBCD-catalyzed degradation (by a recD mutation) should be lethal, because it inactivates both pathways of fork resetting. This prediction was tested and, as expected, the combination of a recA and a recD mutation prevented growth of Inv mutants (InvA recD and InvBE recA recD, Figure 2).

Inactivation of the function catalyzing the first step of fork reversal should restore the viability of Inv recB mutants on LB, by precluding the formation of dsDNA ends. RFR has been shown to be catalyzed by various enzymatic activities. Depending on the cause of replication arrest, RecA, RuvAB, and RecG have been implicated in the formation of reversed forks in vivo or in vitro [6]. The observation that the Inv recA mutant is killed by a recD
mutation indicates that a dsDNA end is still formed in the absence of RecA and that RecA is not the enzyme responsible for fork reversal. As shown in Figure 2, in the absence of either RuvAB or RecG, Inv recB and Inv recA recD mutants remain lethal on LB, indicating that fork reversal still occurs (Inv recB ruvAB, Inv recD recA ruvAB, and Inv recB recG, Figure 2). Inv recB mutants also remained LB sensitive in the absence of both RuvAB and RecG (Inv recB ruvAB recG, Figure 2), excluding a redundant function for these two enzymes. Control Inv recA ruv and Inv recD ruv mutants remained resistant to LB. Therefore, either RFR is not catalyzed in Inv mutants by the RecA, RuvAB or RecG enzymes, or in their absence a redundant, yet unknown enzyme can still catalyze the reaction. High levels of positive super-coiling can promote RFR in vitro [41], and could conceivably accumulate at sites of replication-transcription collisions, if the activity of gyrase (the enzyme that removes positive super-coils) was limiting in vivo. However, increasing (by inserting a gyrase-specific hotspot sequence at the collision site), or decreasing (by a gyrBts mutation) gyrase activity did not affect the viability of any Inv recombination mutant (our unpublished results), which renders unlikely a spontaneous reaction driven by positive super-coiling.

Fork cleavage at inverted rrnA in the InvA recBC mutant

According to the RFR model, the dsDNA ends that are formed at blocked forks are converted into one-ended double-strand breaks (DSBs) by the action RuvABC in the absence of RecBC (Figure 1A, pathway E). To ensure that the RecBC requirement results from fork breakage at the inverted rrn genes, we performed a molecular analysis of the region of replication-transcription collision. I-SceI sites were introduced into the chromosome on both sides of the inverted regions (Figure 1B). Chromosome breakage at rrnA should cleave the 800 kb I-SceI fragment into two fragments of about 400 kb, whereas fork breakage is expected to produce only the origin-proximal one of these two fragments (Figure 1B; Figure 1A, pathway E). We analyzed the I-SceI cleavage products of the InvA recB chromosome by pulse field gel electrophoresis...
(PFGE) and Southern blotting, prior to and after a shift to LB, using a probe that hybridizes with the origin-proximal part of the I-Sce fragment carrying the inversion (Figure 3A, 3B). The intact I-Sce fragment observed in MM disappeared after a shift to LB, while a ≈400 kb fragment appeared in 1 hr and seemed to be converted to DNA fragments of smaller size with time, with the accumulation of a fragment of ≈300 kb in three hours (Figure 3A, 3B). The length of the DNA fragment observed after 1 hr of incubation in LB (≈400 kb) corresponds to the distance between the I-Sce site and the inverted rmd operon (Figure 1B). However, its conversion to smaller fragments at later times suggests that this DNA fragment is degraded in vivo. In E. coli, one of the main exonucleases besides RecBCD is the RecJ exonuclease, which degrades 5’-ended single-stranded DNA produced by the action on dsDNA ends of RecQ or another helicase [42]. We tested whether the I-Sce-rmd fragment of ≈400 kb was being degraded by RecJ and observed that indeed inactivation of RecJ prevented most of the conversion of this fragment to smaller ones (Figure 3A, 3B). This result shows that RecJ degrades dsDNA ends made by breakage at rmd in the InvA recB mutant. No production of linear DNA in vivo could be detected with control strains (non-inverted recB mutants, InvA RecBCD<sup>+</sup> strains, Figure S1).

In order to determine whether the origin-distal part of the I-Sce fragment is also produced by cleavage at rmd, the same membranes were hybridized to a probe within this region. The only fragment detected was in the intact I-Sce fragment (Figure 3C), which, as seen with the origin-proximal probe, decreased in intensity with time. As previously observed for other fork-breakage reactions [43], the absence of detectable origin-distal fragment is consistent with the conclusion that the origin-proximal fragments result from fork breakage (one-ended break) and not from a bona fide DSB (two-ended break) at rmd.

Our attempts to quantify the bands produced by fork cleavage at rmd provided highly variable results, owing partly to the nearly full disappearance of the intact I-Sce fragment, an unexpected observation, and partly to the fact that a large fraction of the probed DNA remained trapped in the wells. Non-migrating DNA may result from partial cleavage and/or from the presence of structures blocking DNA migration (forks, HJ, replication bubbles), thus contributing to the disappearance of the intact I-Sce fragment upon growth in LB. In order to determine whether the non-migrating DNA is trapped in wells by unresolved recombination intermediates, we analyzed chromosome breakage in the InvA recA recD mutant. A large amount of DNA fragments smaller than the intact I-Sce fragment was detected in the InvA recA recD mutant (Figure 3D, 3E), in contrast with in the non-inverted recA recD control strain (Figure S1). The level of non-migrating DNA remained very high and the intact I-Sce fragment disappeared when cells were propagated in LB, still preventing quantification (Figure 3D, 3E). This observation suggests that non-migrating DNA might be trapped in wells owing to the presence of replication intermediate structures. Interestingly, growth of the InvA recA recD mutant in LB triggered the appearance of a DNA band that hybridized specifically with the origin proximal probe, and was not the ≈400 kb fragment expected from breakage at rmd but the smaller ≈300 kb one, observed in the recBC mutant in the presence of the RecJ exonuclease (Figure 3D, 3E). Since in a recA recD context, RecBC and RecJ are active, this ≈300 kb fragment, which is not observed in a recB recJ context, is likely to result from the degradation by the combined action of the RecBC helicase and the RecJ exonuclease of a dsDNA end produced by fork cleavage at rmd.

**Fork cleavage at the inverted rme locus in the InvBE recBC mutant.**

To determine whether fork breakage at the inverted rmd locus is specific for this ribosomal operon, we analyzed the formation of linear DNA in the inverted region of the InvBE recB mutant by I-Sce enzyme. When the InvBE recB recJ mutant was propagated in LB medium, a fragment of about 616 kb that hybridizes with the origin-proximal probe was observed, as expected from fork breakage at rme (Figure 4A, 4B). In the InvBE recB mutant, this
A ~616 kb fragment was transformed into a smear, owing to RecJ-mediated DNA degradation (Figure 4A, 4B). The corresponding origin-distal fragment of about 180 kb was not observed using an origin-distal probe, supporting the conclusion that the origin proximal fragments result from fork breakage and not from a bona fide DSB (Figure 4C). In the InvBE mC recD mutant, the origin-proximal probe revealed a smear of fragments smaller than 616 kb and two main fragments of ~400 kb and ~300 kb (Figure 4D, 4E), which presumably result from degradation of the ~616 kb fragment by RecBC and RecJ as they are not observed in an InvBE mB mCJ mutant. As in InvA mutants, the disappearance of the intact I-SceI band and the high level of non-migrating I-SceI fragments prevented the quantification of linear DNA species. No production of linear DNA in vivo could be detected with control strains (non-inverted recB or recA recD mutants, InvBE RecBCD’ strains, Figure S1).

rm genes are a barrier to DNA degradation

We noted that the ~300 kb and the ~400 kb fragments observed in the InvBE recA recD mutant have a size corresponding to the distance between the I-SceI site and the normally oriented rmc and rmA operons, respectively (Figure 1B). We hypothesized that the product of fork breakage at the inverted rmC locus might be degraded until the first non-inverted rm is encountered by exonucleases. This DNA degradation is not observed in a recB recJ mutant, is slow in a recB recD mutant where it is mainly catalyzed by RecJ (it does not reach rmA, 231 kb away, in two hours), and is more efficient in a recA recD mutant, where the helicase RecBC and RecJ exonuclease are both active (Figure 4). To test the hypothesis that rm genes could be a barrier to DNA degradation, membranes such as the one shown in Figure 4E were hybridized with probes located about 5 kb apart, immediately upstream or downstream of rmc and rmA (Figure 5, only the MM and LB 2 hours lanes are shown, the kinetics of appearance of bands is shown Figure 4E). The ~300 kb fragment hybridized with the rmC promoter region but not with the rmC terminator region, indicating that it ends within rmC (Figure 5, probes 2 and 3). Similarly, the ~400 kb fragment hybridized with the rmD promoter region but not with the rmD terminator region, indicating that it ends within rmD (Figure 5, probes 4 and 5). These results indicate that linear DNA ending in rm sequences accumulates after DNA breakage, suggesting that degradation of linear DNA is slowed down by the encounter with rm sequences. In the mC mD context, DNA degradation reaches rmA, 231 kb away from the inverted rmE in 1 hr, and rmC, 325 kb away, in 2 hours (Figure 4D, 4E).

In the InvA genome, NdI restriction produces a 208 kb fragment carrying rmC (NdI kb 3774 to 3982, Figure 1B), and degradation of the origin distal part of this DNA fragment until rmC is expected to produce a 171 kb DNA fragment (NdI kb 3774 to rmC, Figure 1B). Actually, when the InvA recB mutant was grown for 2 hrs in LB, an additional ~170 kb fragment was observed after NdI restriction, compared to the restriction profile of chromosomes extracted from the same cells grown in MM (Figure 6A). This DNA fragment hybridized only with a probe adjacent to the rmC promoter and not with a probe adjacent to the rmC terminator sequence (Figure 6B, 6C). This result indicates that the fragment ends within rmC, which suggests that DNA degradation is slowed down in rmC in InvA recB cells. This NdI fragment corresponds to the ~300 kb fragment observed after cleavage by I-SceI (Figure 1B), and indicates that DNA 93 kb in length, the distance between rmC and the inverted rmA, is degraded in 2 hours by RecJ in a recB context, and in 1 hour by RecJ and RecBC in a recA recD context (Figure 3). No 171 kb DNA fragments were observed with control strains (Figure S1).

DNA breakage at an inverted rm locus is RecA-independent and partly RuvABC-dependent

The above experiments indicate that following replication arrest at an inverted rmA locus, the products of fork breakage are

![Figure 4. Chromosome breakage in InvBE recB and InvA recA recD mutants.](image)

Chromosomes of the indicated InvBE mutants, grown in minimal medium (MM) or for 1, 2 or 3 hours in LB, were cleaved with the l-SceI enzyme and fragments were separated by PFGE. A. Ethidium bromide stained gel with l-SceI cut chromosomes from InvBE recB and InvBE recB recJ mutants. First lane Saccharomyces cerevisiae chromosome ladder, relevant sizes are indicated on the left. B. Southern blot of the gel shown in A using the origin-proximal probe in the 800 kb l-SceI fragment, both the intact fragment and fragments of smaller sizes hybridize with the probe. C. Southern blot of a gel made with the InvBE recB l-SceI cut chromosomes, using the origin-distal probe in the 800 kb l-SceI fragment, only the intact l-SceI fragment hybridizes with the probe. D. Ethidium bromide stained gel with l-SceI cut chromosomes from the InvBE recA recD mutant. E. Southern blot of the gel shown in D using the origin-proximal probe in the 800 kb l-SceI fragment, both the intact fragment and fragments of smaller sizes hybridize with the probe. F. Schematic representation of the different DNA fragments. The triangles represent the three rm operons as indicated, the black circle represent oriC and the little bars above the lines represent the position of the origin-proximal (left) and origin-distal (right) probes.

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converted by DNA degradation, in the absence of the exonuclease V activity of RecBCD, to smaller chromosome fragments, most of which have a specific length and can be analyzed by NotI restriction enzyme digestion of chromosomes. As the dramatic loss of the intact band was not observed after NotI cleavage, in contrast to I-SceI cleaved chromosomes, we used Southern blots of NotI fragments to quantify the linear DNA resulting from breakage at the inverted mrl locus, at different times after a shift to LB and in different recombination mutants. The intensities of bands corresponding to the 208 kb NotI fragment (i) trapped in wells, (ii) intact (208 kb), or (iii) degraded to mrc after fork breakage at InvA (171 kb), were quantified by Southern hybridization. The proportion of DNA present in each of these three bands was calculated and averages from 3 to 5 experiments are shown in Figure 6E and Table S2. In the InvA mrc mutant, the proportion of the 171 kb DNA fragment produced by fork breakage and DNA degradation up to mrc increased from 9% in MM to 27%±5%, and 37%±3%, after two and three hours in LB, respectively. In the InvA mrcD mutant, this proportion increased to 20%±8% after 1 hour in LB and 30%±5% after two hours, and then decreased, possibly because DNA degradation progresses beyond mrc. The 171 kb DNA band appeared earlier on gels in a mrcD context than in a mrc context, probably because DNA degradation is more rapid in the former mutant, owing to the combined action of RecBC and RecJ. The observation of a similar efficiency of fork breakage in InvA mrc and in InvA mrcD cells confirms that fork breakage is independent of RecA. As expected, the 171 kb fragment was not observed in control strains: recombination mutants without chromosome inversion, a recombination proficient InvA mutant, and an InvA mutant in which only mrc or only mrcD is inactivated (data not shown; Table S2).

It should be noted that, given the absence of the origin-distal dsDNA end at the replication-transcription collision site (Figure 3G, Figure 4C), the origin-proximal dsDNA end is produced by either a reaction where only one of the two replicated chromosome arms at the fork is broken, or by over-replication of blocked replication forks. Either of these reactions will produce an intact copy of the chromosome in addition to the linear DNA fragment interrupted at the position of replication-transcription collision (as in Figure 1A, step E). Therefore, the percentage of 171 kb fragments can be at most 50% of the migrating DNA, assuming 100% breakage. The percentage of chromosomes that have been replicated without DNA damage can be calculated by deducing from the measured proportion of intact 208 kb fragment that of broken DNA (171 kb fragment). These calculations show that most of the DNA is broken upon collision of replication forks with the inverted mrl, in a mrc and in a mrcD context (Table S2).

The RFR model predicts that fork cleavage may be RuvABC dependent (Figure 1A, step E). Since RuvABC does not have any known exonuclease activity, and is not suspected to affect the activity of E. coli exonucleases, we used this assay to compare fork breakage in RuvABC+ and ruvABC mutant cells. The proportion of the 171 kb DNA fragment was nearly two-fold lower in InvA mrc compared to InvA mrcD mutant cells. The proportion of the 171 kb DNA fragment was at most 50% of the migrating DNA, assuming 100% breakage. The percentage of chromosomes that have been replicated without DNA damage can be calculated by deducing from the measured proportion of intact 208 kb fragment that of broken DNA (171 kb fragment). These calculations show that most of the DNA is broken upon collision of replication forks with the inverted mrl, in a mrc and in a mrcD context (Table S2).

Discussion

In this work we analyze the action of recombination proteins at replication forks arrested by a collision with a highly-transcribed, oppositely-oriented mrl operon. We find that RecBC is crucial for replication across this region and that in its absence fork breakage, which is partly RuvABC-dependent, occurs. The RecBC requirement is caused by collisions of replication with RNA polymerases within the inverted ribosomal operon, and does not involve R-loop formation. Furthermore, in the course of this work we observed
that DNA degradation of linear DNA is also arrested by an oppositely-oriented m locus.

RFR occurs at forks arrested by a highly expressed, oppositely oriented rrn operon

Several lines of evidence argue for the occurrence of RFR upon collision of replication with inverted m operons. First, Inv recB and Inv recA recD mutants are sensitive to growth in rich medium, whereas Inv recA and Inv recD mutants are not. Second, the inverted m genes are sites of chromosome breakage, half of which is dependent of RuvABC. We conclude that half of the linear DNA observed in Inv mB cells results from the resolution of reversed forks by RuvABC while the other half results from one or more other processes (Figure 7). We hypothesize that the linear DNA observed in mB ruvAB mutants may result from re-initiation at the chromosome origin oriC. Running of these new replication forks into blocked forks is expected to form linear DNA by copying the newly-synthesized strands of the first forks to the end (Figure 7). Such a run-off reaction has previously been observed at extra Ter sites inserted in the chromosome, and in over-initiation mutants [44,45]. Strains carrying extra Ter sites are only viable if the dsDNA end is repaired by homologous recombination, suggesting that Inv cells that suffer re-replication should also require homologous recombination for replication restart and viability.

Figure 6. Fork breakage is partially RuvABC-dependent in InvA mutants. A. Chromosomes of the indicated InvA mutants, grown in minimal medium (MM) or for 1, 2 or 3 hours in LB, were restricted with the NotI enzyme and fragments were separated by PFGE. A. Ethidium bromide stained gel with NotI restricted chromosomes from InvA recB and InvA recB ruvAB mutants. Fragment sizes are indicated on the left. The star indicates the position of migration of the 171 kb DNA fragment formed by fork breakage and DNA degradation. B. Southern blot of the gel shown in A using the rrnC promoter probe, both the intact 208 kb NotI fragment and fragments of smaller sizes hybridize with the probe (the minor hybridization with the 193 kb NotI restriction fragment may result from co-migration of broken DNA with this fragment). C. Southern blot made with the gel shown in A, using the rrnC terminator probe, only the intact 208 kb NotI fragment hybridizes with the probe. D. Schematic representation of the different DNA fragments. The triangles represent the two rrn operons as indicated, the black circle represents oriC and the bars above the lines show the positions of the probes. E. For each mutant, Southern hybridizations of 3 to 6 gels were quantified, and the percentage of hybridized DNA that remains in wells, that migrates at the 208 kb position and that migrates at the 171 kb position were calculated. For clarity, only the percentages of migrating DNA are shown here (see Table S2 for complete results); light grey, 208 kb fragment (intact), dark grey, 171 kb fragment (resulting from fork breakage and DNA degradation up to rrnC). Vertical bars indicate standard deviations. doi:10.1371/journal.pgen.1002622.g006
Re-replication in Inv mutants may not frequent enough to render RecA essential for growth, probably owing to a significant proportion of blocked forks processed by RFR (Figure 7). Replication re-initiation at oriC might explain the high level of trapped I-SceI fragments in LB, a RecA-independent phenomenon that was less important with the smaller NotI oriC-carrying fragment.

DNA degradation is slowed down by the encounter of rrn operons

We observed that the dsDNA fragments detected in PFGE are subjected to DNA degradation by RecJ, and this reaction is more efficient in the presence of the RecBC helicase. Degradation of dsDNA ends and homologous recombination catalyzed by the combined action of RecBC and various exonucleases including RecJ, have been reported previously in a recD mutant [46,47]. The reaction observed here is slow, on average around 3 kb per minute in a recA recD context where both RecJ and RecBC are active, and around 1 kb per minute in a recB mutant where it depends primarily on RecJ. RecJ is a 5’ to 3’ ssDNA exonuclease, but it can also digest dsDNA in vitro, a reaction that is stimulated in the presence of the RecQ helicase [48]. RecBCD digests dsDNA at a rate close to 1 kb per second [49], which allows it to rescue reversed forks prior to the resolution of the HJ (Figure 1A step D). In contrast, the genetic properties of strains that undergo RFR indicate that RecJ and RecBC do not to rescue reversed forks by DNA degradation. Actually, RecJ digests 3’ ended ssDNA at a rate of about 1 kb per minute in vitro [42]. A RecBCD mutant complex, in which the helicase function of the RecD subunit is inactivated, is three times slower than the intact enzyme in vitro [49]. This may explain why RecJ and RecBC in the absence of RecD, do not catch up with the ResA-migrated HJ prior to resolution by RuvC. In addition RecJ and RecBC are less processive than RecBCD [42,49] and the dsDNA degradation that we observe in this work is likely to result from multiple DNA binding events. Why this DNA degradation slows down at ribosomal operons remains an open question. Some features of the rrn locus, either structural (the operon contains DNA sequences prone to the formation of secondary structures), or functional (the operon might be expressed, even on a broken DNA arm), might trigger the dissociation of RecJ or RecBC from DNA.

Enzymes that catalyze replication fork reversal

We have not identified the enzymatic function(s) that reverse forks in Inv strains, as the inactivation of the enzymes previously shown to reverse forks in vivo or in vitro, RecA, RuvAB or RecG, did not prevent RFR. The Rep, UvrD, and DinG helicases, which act at blocked forks in Inv mutants, are not responsible for RFR since the inactivation of any of them aggravated rather than relieved the requirement for RecBC (our unpublished results). Similarly in a dnaNts mutant, impaired for the β-clamp subunit of DNA polymerase III, forks are reversed by a yet unknown function, indicating that more RFR-catalyzing enzymes remain to be identified in E. coli [50].

Recently, replication fork reversal was proposed to occur in cells where the Rho terminator of transcription is inactivated by a specific agent, bicyclomycin [51]. Inactivating Rho-dependent transcription termination is thought to cause replication-transcription conflicts, and bicyclomycin treatment killed recB mutants but did not kill recA mutants. In the recB mutants, a high level of chromosome breakage was detected, suggestive of the occurrence of RFR. Chromosome breakage was attributed to the resolution by RuvABC of reversed forks, but the role of RuvABC in bicyclomycin-induced chromosome breakage was not tested.
Because the amount of linear DNA measured by PFGE was strongly decreased in a recA mutant, it was proposed that RecA was the enzyme responsible for fork reversal. This interpretation of the data is questionable since in recA cells the very low level of detectable linear chromosomes could reasonably be ascribed to their degradation by RecBCD [22,52]. Actually, we observed here that fork breakage at replication-transcription collision sites is RecA-independent.

RFR is caused by the encounter of replication forks with RNA polymerases

Two kinds of obstacles can impede replication progression across highly transcribed regions: collisions with R-loops, and collisions with DNA-bound, transcribing (or arrested) RNA polymerases. R-loops have been shown to cause replication arrest in several organisms and to stimulate DNA rearrangements, often associated with replication fork blockage ([33,34] and Ref therein). However, R-loops are unlikely to be the cause of replication fork arrest and reversal in Inv recB mutants, because over-expression of RNaseH does not suppress the sensitivity to rich medium of these strains. DinG plays two roles in Inv mutants, as it participates to RNA Pol removal together with Rep and UvrD, and it is the helicase responsible for the removal of R-loops [8]. In vitro studies showed that DinG can remove R-loops by directly recognizing them, independently of the presence of a replication fork [53]. The lack of effects of RNaseH over-expression in the Inv recB mutants leads us to conclude that the presence of DinG actually prevents the accumulation of R-loops in these mutants, and, in turn, that forks are mostly arrested at highly expressed rrn genes by trains of RNA polymerases transcribing the rDNA.

The occurrence of RFR at inverted rrn genes in is agreement with the idea that replication arrest is mainly due to collisions of replication forks with RNA polymerases in a rep single mutant, where RFR was first reported. However, in the rep mutant the conversion of forks into reversed forks was partly catalyzed by RuvAB and partly by an unidentified function [56]. Here we have no evidence for RuvAB catalyzing some of the RFR reactions, since inactivating ruvAB does not rescue the Inv recB or Inv recA recD mutants, possibly because the alternative function can reverse all forks at the inverted rrn locus in the absence of RuvAB.

RFR precedes and may facilitate the action of accessory replicative helicases

We have shown that after replication-transcription collisions, replication restart requires the action of the accessory replicative helicases Rep, UvrD and DinG, which presumably remove RNA polymerases from DNA [8]. The occurrence of RFR indicates that these helicases do not act first, since their action would allow forks to progress unimpeded across the inverted rrn, preventing RFR. In other words, why would RecBC be essential for growth in the presence of Rep, UvrD and DinG, if these helicases could directly act at blocked forks to remove obstacles? Therefore, we propose that RFR takes place first and that helicases act at forks that are reset after reversal (Figure 7). We can envision different reasons why RFR would take place first: either the enzyme that promotes the reaction may have more affinity for blocked forks than the accessory replicative helicases, or there may be too many RNA polymerases on highly expressed rrn operons for the helicases to deal with them within the time frame that is required to initiate RFR, or RFR may be required for the action of these helicases. In support for the latter hypothesis, UvrD was previously shown to act in conjunction with RecBC in two cases of replication fork restart. Firstly, UvrD acts together with homologous recombina-

tion for the removal of Tus from extra Ter sites. UvrD is then required for viability, and does not directly remove Tus from Tus/Ter-blocked forks, since it does not bypass the need for homologous recombination [57]. Secondy, UvrD is essential for viability in the rep mutant, where it necessarily acts after RFR, since it does not bypass the need for RecBC. These observations suggest that UvrD might more easily find its target on PriA-dependent restarted forks formed after homologous recombination, or after degradation of reversed forks. At forks blocked by an inverted rrn locus, the concerted action of two accessory helicases is required for restart, which are Rep, and either UvrD or DinG [8,32]. However, in contrast with UvrD, Rep acts without homologous recombination or RFR, since it acts in wild-type E. coli where RecBC is not essential for viability (RecBC becomes essential only in its absence, when UvrD is required). Rep may be directly targeted to blocked forks by its interaction with the DnaB helicase [33,35]. We propose that RFR occurs at forks blocked by an inverted rrn locus to promote the action of UvrD or DinG, required here in addition to Rep (Figure 7). Forks that have been reversed, and then converted back to a fork structure either by homologous recombination or by degradation of the DNA double-strand end in the reversed fork may be easier targets for UvrD and DinG than the original blocked forks. Although the molecular mechanism by which fork reversal facilitates replication restart is not known at present, we can speculate that replication fork reversal may trigger the disassembly of the blocked replisome and thereby facilitates access to DNA for the accessory helicases, and/or allow the targeting of these helicases to restarting forks by some interaction with a recombination or replication restart protein.

Materials and Methods

Strains and constructions

Strains and plasmids used in this work are described in Table S3 (Ref. in Text S1). They were constructed by P1 transduction. The recB mutation was introduced in the presence of the wild-type recCD genes carried on an IPTG-dependent plasmid pAM-RecBCD. A pAM-RecA plasmid was used for propagation and P1 transduction of recA mutants. Plasmids were cured prior to each experiment by growing cells in the absence of IPTG and plasmidless colonies were isolated on minimal medium (MM). All other mutations were directly introduced in the Inv mutants by P1 transduction on MM. For insertion of the I-SceI site into the chromosome, the following double-stranded sequence 5’ GCATGCATTGGAAGGTAATTACTAGGATATGAT’ carrying the I-SceI cleavage site (in italics) was inserted between the CdtA and SphI sites of plasmid pKD3 [50]. Then the site was amplified by PCR together with the adjacent FRT::CmR-FRT region of pKD3 and the PCR product was inserted into the chromosome as described [58].

Measures of viability

Appropriate dilutions of overnight cultures in MM were plated on MM and LB plates. Plates were incubated at 37°C and the number of colony forming units was counted after 16–24 hours of incubation for LB plates and after 48 hours of incubation for MM plates.

Fork breakage analysis

For each experiment, freshly isolated colonies of recB or recA recD mutants cured of the pAM-RecBCD plasmid were used for overnight cultures. However, for some mutants that grow slowly in MM, overnight cultures of cells carrying pAM-RecBCD were grown in the absence of IPTG. These cultures were plated on
plates with or without Ap and IPTG and they contained less than 3% of plasmid carrying cells. This protocol was used for the following mutants: InvBE and InvA recD recJ, InvBE and InvA recD recAB, and InvA recD recAB. Plugs preparation, NotI restriction, and gel of NotI-digested DNA were performed as described in [8]. Cleavage of DNA by I-SceI was performed in plugs as recommended by the supplier, with 1 hr incubation at 37°C in the presence of 2.5 units of enzyme. PFGE of I-SceI restricted chromosome was as for NotI, but with a ramp of pulses from 40 s to 70 s and migration for 18 hours. Transfer, hybridization, probe preparation, and quantification with Image Quanta were performed as described in [8]. In Figure 3 and Figure 4 the origin proximal probe is in the slp gene and the origin distal probe in the yieP gene. Probes used in Figure 5 are in the following genes: probe 1 - origin-proximal probe: slp gene, probe 2 - rnc promoter probe; yieP gene, probe 3 - rnc terminator probe: hfrR gene, probe 4 - rmd promoter probe: hemG gene, probe 5 - rmd terminator probe: mobA-yihE genes, probe 6 - origin-distal probe: cygC gene. Probes used in Figure 6 are rmc promoter probe: yieP gene, and rnc terminator probe: hfrR gene.

Supporting Information

Figure S1 Control strains do not produce linear DNA when shifted to rich medium. For all experiments shown in Figure 3, Figure 4, Figure 5, Figure 6, plugs of a control strain expected to show no fork breakage (a non-inverted recB or recD mutant, which InvA or InvBE RecBC+ mutant) were prepared in parallel with plugs of Inv recBC or Inv recD mutants. Each PFGE gel, and consequently each membrane used for Southern blotting, carried at least one such control, that indeed showed no fork breakage, as shown here. Representative examples of these control lanes are shown here. Top panels I-SceI-treated chromosomes from: A - Non-inverted strains, Rec+ (wt, JJC5329), recB (JJC5326) and recD (JJC5312 cured of pAM-RecA+); B - InvA (JJC5391), C - InvBE (JJC5352), InvBE recD (JJC5390) and InvBE recA (JJC511 cured of pAM-RecA+). Bottom panels NotI-treated chromosomes from: D – non inverted strains, recB (JJC5326) and recD (JJC5319). (DOC)

Table S1 Inactivation of the yieP gene does not affect the viability of Inv mutants.

Table S2 Quantification of fork breakage.

Table S3 Strains and plasmids.

Text S1 Supplementary references.

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Author Contributions

Conceived and designed the experiments: ALDS HB SD BM. Performed the experiments: ALDS HB SD. Analyzed the data: ALDS HB SD BM. Wrote the paper: ALDS HB SD BM.
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