Replication fork collapse at a protein–DNA roadblock leads to fork reversal, promoted by the RecQ helicase

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Summary
Proteins that bind DNA are the cause of the majority of impediments to replication fork progression and can lead to subsequent collapse of the replication fork. Failure to deal with fork collapse efficiently leads to mutation or cell death. Several models have been proposed for how a cell processes a stalled or collapsed replication fork; eukaryotes and bacteria are not dissimilar in terms of the general pathways undertaken to deal with these events. This study shows that replication fork regression, the combination of replication fork reversal leading to formation of a Holliday Junction along with exonuclease digestion, is the preferred pathway for dealing with a collapsed fork in Escherichia coli. Direct endo-nuclease activity at the replication fork was not observed. The protein that had the greatest effect on these fork processing events was the RecQ helicase, while RecG and RuvABC, which have previously been implicated in this process, were found to play a lesser role. Eukaryotic RecQ homologues, BLM and WRN, have also been implicated in processing events following replication fork collapse and may reflect a conserved mechanism. Finally, the SOS response was not induced by the protein-DNA roadblock under these conditions, so did not affect fork processing.

Introduction
During DNA replication, the replication machinery (replisome) can arrest due to impediments on the DNA such as lesions or nucleoprotein blockages. Removal of bound proteins that the replisome itself fails to displace can be carried out by accessory helicases: in Escherichia coli these are Rep, UvrD and/or DinG (Guy et al., 2009; Boubakri et al., 2010). However, even with the full complement of these helicases, protein roadblocks are still found to be the most common obstacle to replisome progression, especially RNA polymerase (Gupta et al., 2013). Encounter with a protein roadblock can lead to the dissociation of the replisome, and the frequency with which this happens is indicated by the central role of PriA/ PriC restart pathways in bacterial cell viability (Marians, 2000). If the blockage is not removed, DNA replication is not able to continue to completion and the cell will not survive. The processing of these stalled forks is likely to be relatively frequent with most or all replisomes predicted to stall during the cell cycle (Cox et al., 2000; Cox, 2001). Bacterial replisome dissociation has recently been reported to be occur at a frequency of about five events per replisome, per cell cycle (Mangiameli et al., 2017).

The DNA at a replication fork that has stalled upon encounter with a protein block can be processed by a number of possible pathways. Endonucleases can cut the forked DNA, producing a double strand break, followed by homologous recombination that restores DNA integrity (Seigneur et al., 1998). Alternatively, exonucleases can act to degrade the nascent leading and lagging strands, moving the Y-shaped branch point away from the site of blockage (Dillingham and Kowalczykowski, 2008). Finally, replication fork reversal (RFR) can occur, whereby the leading and lagging nascent strands separate from their respective template strands and anneal to each other, concurrent with the two template strands also re-annealing [Reviewed in (Atkinson and McGlynn, 2009)]. This leads to the formation of a four-way DNA structure called a Holliday junction (HJ) that is the substrate for proteins in homologous recombination pathways. This HJ has one arm that has free DNA ends that itself can be acted upon by exonucleases whilst the other three arms are continuous DNA. Despite RFR being observed following replication fork-transcription collisions (De Septenville et al., 2012), the relative frequency, or preference for, RFR has by no means been investigated thoroughly. DNA breakage or the recruitment of RecA to ssDNA at the stalled fork may lead to SOS induction (Phizicky and Roberts,...
1981). However, the SOS response is only expected to be a major influence in incidences of prolonged DNA damage, with high levels of unresolved ssDNA and when the recombination pathways are ineffective at processing blocked forks [reviewed in (Friedberg et al., 1995)]. RecA acts in the SOS response as a co-protease to cleave the LexA repressor, inducing SOS [reviewed in (Cox, 2007)]. The RecA protein has been proposed to play a role in RFR, employing its strand exchange capacity; this most readily explained as a RecA filament, bound to ssDNA on the lagging strand template of a replication fork, which catalyses invasion into the leading strand duplex, displacing the nascent strand and forming a reversed fork (Seigneur et al., 2000). RecA-mediated RFR seems to occur only under specific conditions and is restricted in the presence of the single-stranded DNA binding protein (Robu et al., 2001; Robu et al., 2004; Gupta et al., 2014b). Other proposed mechanisms that lead to RFR in bacteria, including the involvement of the homologous recombination protein RecA (Seigneur et al., 2000), are positive supercoiling ahead of the replication fork (Postow et al., 2001) and a number of possible helicase/translocase proteins, including the branched DNA structure binding proteins, RuvAB and RecG (Seigneur et al., 1997; McGlynn and Lloyd, 2000; Abd Wahab et al., 2013; Gupta et al., 2014a).

The DNA binding protein RuvA has high specificity towards branched DNA structures and loads the hexameric RuvB onto this DNA to perform the helicase and branch migration activities of the complex (Iwasaki et al., 1992). When the endonuclease RuvC is also present, the complex is able to resolve the HJ as well as migrate it (Shah et al., 1997). The monomeric superfamily 2 (SF2) helicase and nucleic acid translocase, RecG, has been shown to be able to migrate HJs but can recognize a wider range of substrates than RuvA. RecG binds dsDNA and has been shown to unwind HJs, D-loops, R-loops and partial fork structures [reviewed by (Lloyd and Rudolph, 2016)]. RecG has a wedge domain that confers its ability to bind to these DNA structures at the ssDNA branch point and two helicase domains that drive the unwinding as the protein translocates (Mahdi et al., 1997; Singleton et al., 2001). The growing evidence for the role of RecG in RFR and the finding that RuvABC-mediated RFR is only required in the absence of certain replosome components has subsequently meant RecG has replaced RuvAB as the major protein implicated in RFR (Baharoglu et al., 2006; Abd Wahab et al., 2013; Manosas et al., 2013). It has, therefore, been suggested RuvAB may only be required to branch migrate the HJ once RecG has performed the initial RFR (Buss et al., 2008; Abd Wahab et al., 2013). A study of the proteins responsible for RFR after replication-transcription collisions failed to identify the protein candidate responsible for catalyzing RFR in the absence of RecA, RecG and RuvAB, all three proposed RFR catalysts (De Septenville et al., 2012).

RecQ helicases, first identified in E. coli but conserved from bacteria to humans, are monomeric members of the SF2 superfamily (Nakayama et al., 1984; Nakayama et al., 1985; Zhang et al., 2006). It can initiate unwinding from a 3’ ssDNA overhang, dsDNA ends, or from internal dsDNA (Umezu et al., 1990; Umezu and Nakayama, 1993; Harman and Kowalczykowski, 2001). RecQ is a multifunctional helicase with roles in homologous recombination and suppressing non-homologous or illegitimate recombination. RecQ has been shown to act with TopoII as a mechanism for changing catenation of DNA, to counteract illegitimate recombination events and to resolve converging replication forks (Harmon and Kowalczykowski, 1998; Suski and Marians, 2008). During homologous recombination the helicase is predominantly associated with the RecFOR recombination pathway employed to process ssDNA gaps in the DNA commonly caused by UV irradiation (Courcelle and Hanawalt, 1999; Morimatsu and Kowalczykowski, 2014). In this pathway, RecQ translocates along the ssDNA gap and unwinds dsDNA, further enlarging the single stranded region so that other proteins can gain access to the DNA damage, including the RecFOR proteins that can then load RecA. In vitro analysis of the activity of the human RecQ helicases BLM and WRN on model fork structures determined that they are both able to reverse forks into HJs (Machwe, Xiao et al., 2006; Ral et al., 2006). Interestingly, BLM and WRN can also promote the opposite reaction, un-reversing HJs back into forks (Machwe, Lozada et al., 2006). Another human RecQ homologue, RECQ1, has been shown to be important in vivo for the restart of replication forks that have been reversed and catalyses restoration of forks from their reversed state (Berti et al., 2013). However, E. coli RecQ is yet to be implicated in RFR in vivo.

One of the major impediments to studying RFR in vivo has been the combination of a large chromosome and a rapid replosome: E. coli contains a genome of ~4.6 Mbp and the replosome moves at a rate close to 1 kb s⁻¹ (Marians, 2000). A system has been developed whereby a site-specific road block to DNA replication can be induced at a known position in the chromosome using a transcriptional repressor (TetR) bound to an array of operator sites within the E. coli chromosome (Poszos et al., 2006; Mettrick and Grainge, 2016). Addition of a temperature sensitive allele of the replicative helicase (DnaBts) allows the rapid inactivation of the replosome across a population of cells, and the replication forks are processed by the cell within 5 min (Mettrick and Grainge, 2016). Furthermore, the disappearance of the forked DNA structure from the array region coincided with the visualization of HJs upstream of the array suggesting RFR had taken place in a sizeable proportion of cells (Mettrick and Grainge, 2016). The
disappearance of Y-shaped DNA will be used here as a definition of replication fork collapse; whether or not this is accompanied by partial or complete dissociation of the replisome is unknown. The regression of the replication fork away from the site of DNA damage or protein block allows repair proteins or accessory helicases to access the DNA and resolve the problem. The upstream HJ that is formed may be processed in a recombination-dependent or independent manner to restore a replication fork [reviewed by (Atkinson and McGlynn, 2009)]. If the blockage is removed, replication is able to restart using the reformed fork structure onto which replisome reloading takes place (Possoz et al., 2006), most likely in a PriA-dependent manner. Indeed, replication was observed to restart and proceed through the array in the vast majority of cells within 5 min of the addition of anhydrotetracycline.

In this report, a site-specific replication block was established and characterized at the lac locus, approximately midway round the right replichore on the E. coli chromosome. A dnaBts allele was introduced to be able to trigger replication fork collapse by shifting to a non-permissive temperature, as has previously been shown to be effective in a related strain that has the tetO array at a different locus (Mettrick and Grainge, 2016). The array produces an artificial blockage on the chromosome, and one that is not removed by accessory helicases, such as Rep or UvrD. However, the processing events that the cell uses at the Y-shaped forked DNA following collapse of the replisome at a protein block are not likely to be different between a single protein block (for example, a collision with RNA polymerase) and the presence of the large array ahead of the replication fork; both will prevent access to the dsDNA ahead of the fork. However, some caution in interpreting the results presented is probably warranted and the processing events may well be different in response to replication fork stalling caused by DNA damage as opposed to a bulky protein blockage. The precise relative disposition of the leading and lagging strands and the accessibility of the DNA ahead and behind the fork will dictate what pathways are favoured, and the results presented here are specific to DNA processing at a large protein-DNA roadblock.

This site-specific replication blockage system was used to examine the process of RFR, and the relative contributions of candidate proteins. RFR is seen to play more minor role in RFR. However, the data suggest that RecG is involved in migrating the HJ formed by RFR back into a forked DNA structure that can be used for replication restart; in the absence of RecG HJs are much more prominent and are seen to persist. The induction of the SOS response was only observed to begin after 4 h of replication fork blockage, implying that it does not affect fork processing during the standard experimental timeframe used here.

**Results**

**Visualizing replication fork reversal by inducing replisome collapse**

Previous work has established that a fluorescent repressor/operator system (FROS) using an array of tetO sequences integrated 16 kb anticlockwise from oriC can block replication when the cognate repressor (TetR-YFP) is overproduced from a plasmid (Possoz et al., 2006; Mettrick and Grainge, 2016). In this study, a similar tetO array is utilized, inserted at the lac region roughly half-way between the origin and the terminus on the clockwise replichore (Fig. 1A). The tetR-YFP repressor gene, housed in the pKM1 plasmid, is controlled by an arabinose promoter (Fig. 1A). Growth experiments were carried out to verify that the roadblock was functional at the new position (Fig. 1). The induction of TetR-YFP with arabinose for 2 h at 30°C resulted in the vast majority of cells (98%) containing one fluorescent focus per cell, determined microscopically (Fig. 1B). Cells with a single focus, representing the tetO array, have not duplicated the array region. When replication is not blocked in the unsynchronized culture, there is a mixed population of cells with one, two and sometimes more than two foci. A predominance of cells with a single focus point indicates replication has been arrested across the population, and is used in subsequent experiments as an indication that replication blocking has been successfully established prior to further experimentation (Possoz et al., 2006; Mettrick and Grainge, 2016). Cells that had been grown with arabinose to induce the DNA replication roadblock had ~1000-fold decrease in viability compared to untreated cells (Figs 1C and S1).

To assess whether the established replication block could be removed to allow the resumption of replication, anhydrotetracycline (AT) was added to blocked cells and cultures were reanalysed after a 10 min period. 80% of the cells now contained two or more fluorescent foci, indicating that the array had been duplicated and that the two copies had segregated from each other (Fig. 1B). Given the position of the array, the 10 min AT exposure does not allow time for a newly formed fork to
start from oriC and progress to the array position. Thus, the multiple foci are most likely produced exclusively from restarted forks. The viability of these cells was restored to initial untreated levels, confirming that replication was able to restart throughout the population (Figs. 1C and S1). These results confirm that the tetO array, incorporated at lac, is an effective blockage to replication when the repressor is overproduced, and that the roadblock is reversible with addition of AT. It should be noted that the level of AT added in these experiments (~20 μM) greatly reduces the affinity of TetR for the tetO array but does not completely abolish TetR binding, allowing foci to still be observed in these cells but with a higher background fluorescence from the unbound TetR-YFP molecules (Possoz et al., 2006). However, replication forks are now able to move through the array.

The dnaBts allele was incorporated into the tetO array-carrying strain and assessed in comparison to a WT
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strain for its ability to block and restart replication (Fig. 1D and E). It has recently been proposed that the replication fork present at the TetR-YFP roadblock is not stable but has a half-life of 3–5 min (Mettrick and Grainge, 2016). An equilibrium exists between forks that are stalled at the block and ones that have collapsed and are being processed and will subsequently restart leading to their collision with the replication roadblock once again. DnaBts is utilized here to ensure the synchronous dissociation of the replisome across the entire population and to provide a regulatory tool where replication restart is dependent on DnaBts reactivation. The incorporation of dnaBts did not alter the ability of the replication roadblock to function at permissive temperature, and the results were identical to the WT strain (compare Fig. 1B–D); following overproduction of the TetR-YFP repressor 98% of cells had a single fluorescent focus and viability was decreased over 1000-fold. Addition of AT, to release the replication roadblock, led to the rapid restart of replication with 80% of cells containing two or more foci after 10 min, and a complete recovery of viability.

Following validation of the replication block in both strains, the effect of a shift to 42°C, a non-permissive temperature for DnaBts, was investigated. Cells were grown at 30°C and replication was blocked by overproduction of TetR-YFP as before. Each strain was then transferred to 42°C for 1 h, to inactivate DnaB in the temperature sensitive strain. The ability of DNA replication to restart in each strain was then compared by transferring cells back to the permissive temperature of 30°C for 10 min, with or without the addition of AT. In the WT strain a similar pattern was seen to cells grown only at 30°C; the majority of the population had a single fluorescent focus following the temperature shifts indicating replication was still being efficiently blocked, and addition of AT led to ~70% of cells showing two or more foci within 10 min of addition of AT demonstrating effective replication restart (Fig. 1B). Similarly, viability was almost fully recovered by addition of AT (Fig. S1). Therefore, the temperature shift to 42°C had little noticeable effect upon the replication block or replication restart in the WT strain. In the dnaBts strain the 1 h at 42°C did not affect the integrity of the replication blockage as judged by the high proportion of cells with a single focus and the drop in viability when arabinose is present (Fig. 1D and E). Upon addition of AT when the cells were returned to 30°C viability was also seen to recover to WT levels. This demonstrates that transient inactivation of DnaBts is well tolerated and cells recover fully when returned to a permissive temperature and the replication blockage is removed. However, the proportion of cells with two or more foci after 10 min of AT treatment is much lower than seen with WT. This likely

Fig. 2. Replication fork collapse visualized by 2-D agarose gel electrophoresis.
A. DNA structures from indicated conditions for array region in WT and DnaBts strains, visualized by Southern hybridization. Schematic represents array fragment created by digestion with EcoRI (arrows indicate restriction sites).
B. Quantification of the Y-structured DNA in the array region at 30°C (2 h growth with arabinose) and 42°C (an additional 1 h grown with ara).
C. DNA structures from indicated conditions for region upstream of array in WT and DnaBts strains, visualized by Southern hybridization. Schematic indicates upstream fragment created by digestion with HincII (arrows indicate restriction sites). Note that the vertical smear from the linear spot in the first two gels is not consistently present and is probably due to the large amount of DNA and inter-gel variation.
D. Representative image illustrating the various DNA structures detected by the probes. When replication is blocked, forks remain in the array fragment and accumulate at a similar position on the Y-arc. A line signal protruding from the linear DNA and/or a cone shape extending from the very top of the Y-arc represent Holliday junctions (HJ). See also Fig. S2 and Fig.S3. n = 2–3.
reflects the extra time this strain requires to restart replication that may involve the refolding of inactive DnaBts protein or the novel synthesis of the protein. It has been shown that the majority of locations on the E. coli chromosome, including lac, require 7–10 min to visibly segregate from the sister DNA following replication (Nielsen et al., 2006; Joshi et al., 2011). Thus, even a short delay in restarting replication in the dnaBts strain may be sufficient to prevent the replicated sister duplexes from separating from each other, within the resolution limit of the microscope.

The DNA structures present within each strain during the replication block experiment were visualized using 2-D neutral–neutral agarose gels (Fig. 2). DNA from each condition was extracted, digested and electrophoresed. A radiolabelled probe was used to detect either a 5.8 kb array region including 0.6 kb upstream from the beginning of the array, or a 3.3 kb region from 0.5 kb to 3.8 kb upstream of the array (Fig. 2). At 30 °C, both the WT and dnaBts strains showed a strong, localized spot of Y-shaped DNA in the array region after the addition of arabinose, indicating replication fork blockage (Fig. 2A). With a shift to 42 °C for an hour, persistent forked-DNA structures remained in the WT strain consistent with maintaining the equilibrium of blocked forks and fork turnover (Fig. 2A) (Mettrick and Grainge, 2016). The additional hour of blockage and the temperature shift did not significantly alter the replication blockage in the array region of the WT strain. Quantification of the linear and forked DNA signals determined that ~60% of DNA was seen to be Y-shaped at 30 °C; ~70% was as a Y-structure after the hour at 42 °C (Fig. 2B). The region upstream of the array showed that upon replication blockage a clear signal for HJ and Ys could be seen (Fig. 2C), and the levels of these signals did not vary with changes in temperature in the WT strain, suggesting that the replication forks maintain a relatively constant fork turnover rate. Quantification showed ~9% of the DNA was non-linear upstream when replication was blockage and the temperature shift did not significantly alter the replication blockage in the array region of the WT strain. The equilibrium of blocked forks and fork turnover (Fig. 2A) (Mettrick and Grainge, 2016). The additional hour of blockage and the temperature shift did not significantly alter the replication blockage in the array region of the WT strain. 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microscopy, and the population showed a slight increase in red fluorescence detected by the plate-reader (2250 RFU in WT cells, slightly lower than uvrD by 250 RFU, Figs 3 and S4). This indicated that the SOS response was active in some cells. By 6 h, the majority of the cell population were seen to display a red fluorescence signal, and correspondingly an RFU of 7300 was detected using the plate reader (similar to uvrD). The fluorescence detected at 24 h post fork-blockage had increased dramatically (22,000 RFU) though plasmid loss and cell death were observed alongside cells with increased mCherry intensity (Fig. 3). This result was similar to the uvrD 24-h sample (Fig. S4). This level of SOS induction after 24 h of replication fork blockage was slightly greater than that seen 2 h after exposing cells to 10 Jm⁻² of UV (data not shown). As expected, over the time course, no mCherry fluorescence was seen in any recA cells by microscopy (data not shown), and the plate reader detected under 1800 RFU of mCherry (Fig. S4), confirming that the SOS response is not induced in the absence of RecA. The effect upon SOS induction of releasing the replication roadblock by the addition of AT after 2 h of fork blockage was also assessed. No observable SOS induction was seen 2, 4 or 22 h post AT addition in either WT, uvrD- or recA- strains (Figs 3 and S4). This confirms that the SOS response is not induced by production of the replication roadblock within the first 2 h, and thus the initial replication fork processing events observed are not influenced by the SOS response.

Replication fork processing and RFR can be induced across the population by a shift to the non-permissive temperature for the dnaBts strain, and this is observed to occur rapidly (Mettrick and Grainge, 2016). Therefore, DnaBts can be utilized to collapse forks and monitor RFR events over time in a population of cells. We cannot rule out the possibility that prolonged exposure of dnaBts cells to 42°C following the establishment of the replication fork block will induce SOS. However, processing events seen at the early time points after 90 min of inducing the replication roadblock should not be influenced by SOS induction.

RFR is more dependent on RecQ than RecG or RuvABC

To determine the contributions of various proteins to RFR at a nucleoprotein block in vivo, mutations in recG, ruvABC or recQ were introduced in the dnaBts strain containing the tetO array. Once the formation of a replication blockage had been verified by fluorescence microscopy, the cells were shifted from 30°C to 42°C to inactivate DnaBts. The previous experiment established that a strain carrying DnaBts had the majority of its blocked forks reversed out of the array within an hour at the non-permissive temperature (Fig. 2). To more closely investigate the timing of RFR, samples were taken at 15-min intervals in both the dnaBts and dnaBts + helicase mutant strains at 42°C and the DNA structures present in the array region were analysed via 2-D gels. If the Y-structured DNA indicative of a blocked replication fork persists at the array region within one of the strains, it indicates that the mutation is affecting the cell’s ability to process the replication fork. The region upstream of the array was also visualized to see if the observed levels of Y-structures at the array correlated with the presence or absence of upstream DNA structures. In dnaBts cells with replication blocked by addition of arabinose, a shift to 42°C resulted in a drastic loss in Y-structured DNA signal at the 15 min time point; 15% of the DNA remained Y-shaped, from the 50% seen initially (Fig. 4B). The DNA upstream was mostly linear with a low level of Y and HJ DNA, and remained so over the hour (Fig. S3, Fig. 2C).

Following the same procedure to block DNA replication in dnaBts recG cells, a strong Y-shaped forked DNA signal

Fig. 3. SOS induction begins after 4 h of a persistent replication block and increases thereafter. The strain carrying sulAp-mCherry was grown with 0.05% arabinose at 30°C and samples were taken after 2, 4, 6 and 24 h. After the 2 h, a subset of cells were incubated with AT (10 μg ml⁻¹) and samples taken at 2, 4 and 22 h. SOS induction was observed via (A) mCherry fluorescence emission at indicated time points in Relative Fluorescence Units (RFUs) in blocked (+ara) and released (+ara +AT) cells. Average and standard deviation is shown from three biological replicates. B. microscope images at each time-point in replication blocked cells showing an overlay of phase contrast images and mCherry fluorescence. Red cells indicate sulAp-mCherry expression (SOS induction) Scale bar = 4 microns. See also Fig. S4.
was observed indicative of the replication roadblock, but at a significantly lower amount than dnaBts (Fig. 4A and B). Upstream of the array a strong HJ signal is visible, and is reproducibly stronger than in other genetic backgrounds; ~10% of DNA was in HJ conformation whereas none were detected in dnaBts (Figs 4C and S5). At the permissive temperature for DnaBts, an equilibrium occurs with some spontaneous fork collapse, processing and subsequent replication restart (Mettrick and Grainge, 2016). Yet, without RecG there is a marked accumulation of upstream HJ signal, indicating a role for RecG in the timely processing of these HJs to allow replication restart.

Shifting the dnaBts recG cells to 42°C resulted in a reduction in Y-DNA signal over time within the array region as fork-processing occurs, and this was broadly similar to that seen in WT (Fig. 4A and B). However, a small (but significant) increase in the level of Y-shaped DNA remaining was seen compared to dnaBts. Upstream of the replication block, the loss of HJ and Y-shaped signals over the 42°C time-course occurred (Figs 4C and S5), but this occurred slightly more slowly than in dnaBts, where by 60 min all the signal had disappeared (Fig. S3). The higher level of HJ seen may reflect that RFR takes longer to be resolved in a recG mutant with DNA existing as a HJ for a longer time, even in the presence of RuvABC. It is noteworthy that the position of the HJ signal in these and subsequent 2-D gels is that of a so-called ‘X spike’, which is distinctive of a HJ made from two dsDNAs of full length. If degradation on one arm had occurred, or if the HJ was formed by homologous recombination using a broken strand within this region, then it would migrate in the ‘cone signal’ area and not as seen. The HJ is, therefore, distinctive of a RFR event.

The dnaBts ruvABC mutant produced a replication block at a similar level to dnaBts at 30°C (Fig. 4A). Inducing fork collapse at 42°C led to a rapid drop in Y-DNA signal as seen in dnaBts (Fig. 4B). However, after an hour at 42°C a slightly higher percentage of DNA remained as a fork structure, 20% compared to 12% for dnaBts, representing a moderate deficiency in processing the forked DNA. The prominent HJ seen upstream in the recG mutant was not present in dnaBts ruvABC and the non-linear DNA structures that were present were largely processed in the course of the hour at 42°C, similar to dnaBts (Fig. 4C; compare Fig S3–S5). It is noteworthy that this processing of the upstream DNA appeared efficient even in the absence of RuvABC.

The greatest persistence of Y-shaped DNA following a shift to 42°C was seen in the dnaBts recQ mutant. Upon establishment of a replication block at 30°C, 65% of DNA was Y-shaped, a significantly larger proportion than in dnaBts (Fig. 4A). After 15 min at 42°C the DNA remained largely in the forked-DNA structure (Fig. 4A). Quantitatively, forked DNA now made up 44% of the total DNA, signifying that only a third of the initial Y-shaped DNA had been processed. The fork signal persisted even after an hour at 42°C, with 33% of the total DNA still being in a Y-structure (Fig. 4A and B) i.e. roughly half the initial Y-shaped DNA remained after 1 h. Upstream of the replication block, HJ and

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**Fig. 4.** RFR frequency is reduced in dnaBts recG, dnaBts ruvABC and dnaBts recQ mutants. 2-D gel analysis of replication block (+ ara) after 0, 15 and 60 min at 42°C (the non-permissive temperature for DnaBts), following an initial growth with arabinose at 30°C for 2 h.

A. DNA structures within the array region.

B. Percentage of Y-structured DNA within the 5.8 kb array region.

*Denotes significant difference between Y-DNA amount in dnaBts and the mutant at 0 min (P < 0.05). At 15 and 60 min, the percentage of Y-DNA remaining in each mutant was determined and deemed significantly different to that remaining in dnaBts (P < 0.05, *P < 0.01 and **P < 0.001).

C. Replication intermediates detected in the region upstream of the array. n = 3.
Y-shaped intermediates were present at all times; this could reflect that processing of the upstream DNA was also slower in recQ mutants, or that there was a constant low level of processing of the Y-shaped structures at the block to generate more upstream signals that continued over the full hour (Figs 4C and S5). Slower processing of the stalled fork could explain why a higher proportion of Y-structured DNA was initially seen at the array upon blockage at 30°C: it is the result of an alteration of the equilibrium between fork collapse/processing and restart similar to what was proposed for the RecG results above; in recQ the initial fork regression is slowed, whereas in recG HJ migration to allow restart is slowed.

These findings demonstrated that recG, ruvABC and recQ mutants exhibit a diminished ability to reverse replication forks, implying that all three proteins are involved in RFR though to different extents. It also implies that very little direct endonuclease digestion of the Y-shaped DNA occurs, as it is difficult to envisage this being restored to a stable Y-structure at the non-permissive temperature for DnaBts. The greatest deficiency to fork processing was seen in dnaBts recQ indicating that RecQ is a key under these conditions. The majority of processing of the Y-shaped DNA in these experiments occurs by RFR rather than direct nuclease cleavage of the Y-shaped DNA, as seen by the accumulation of HJs upstream. Following inactivation of DnaBts it has been seen that both ExoI and RecJ can contribute to degradation of the nascent DNA strands (Belle et al., 2007), and RecJ activity has been shown to be stimulated by RecQ activity that provides a suitable substrate (Morimatsu and Kowalczykowski, 2014). Therefore, inactivation of RecQ may inhibit processing that leads both to RFR and exonuclease digestion to yield Y-shaped structures upstream.

RecG and RuvABC act in a distinct pathway from RecQ to process stalled forks

To determine whether RecG, RuvABC and RecQ act synergistically to perform RFR, we investigated strains with combinations of these gene knockouts. If these proteins act independently, the absence of multiple proteins should be an additive effect, and the signal indicative of forked DNA that accumulated in the array region during induction of the replication blockage will persist at a higher proportion than in any of the single mutants alone upon DnaBts inactivation.

The dnaBts recGrecQ mutant behaved in a broadly similar way to the dnaBts recQ strain and was impaired in processing of forked-DNA at the array (Fig. 5A and B). The intensity of the Y-DNA signal was unchanged from the 15 to 60-min time points (37%, Fig. 5A and B), meaning the double mutant was marginally more impaired than a recQ mutant alone. The HJs and Y-arcs detected upstream after the initial replication block (0 min) in dnaBts recGrecQ mutant included a prominent HJ signal equating to 5%
of total DNA, like those seen in a recQ mutant (Fig. S5). Shifting the cells to 42°C resulted in a gradual loss of the Y-DNA arc but some persistent HJ (Figs 5C and S5).

Analysis of the dnaBts recQruvABC strain bore a close resemblance to that of dnaBts recQrecG and dnaBts recQ. Once shifted to 42°C the intensity of the forked DNA signal at the block remained relatively high at both the 15- and 60-min marks (Fig. 5A and B). Of the original Y-shaped signal 54% remained at 60 min (Fig. 5B). Upstream DNA signals were also akin to those seen in the dnaBts recQrecG mutant at 15 min, with a moderate increase in Y and HJ signals, but these then became fainter after an hour (Fig. 5C; Fig. S5). The similarity in the signals seen in both recQrecG and recQruvABC could mean that that RecG and RuvABC contribute equally to RFR, or could act in the same pathway together. However, RecQ’s contribution to RFR appears to far outweigh either RecG or RuvABC.

When dnaBts recGruvABC cells were grown at 30°C and the replication roadblock was induced, ~45% of the total DNA was seen to be Y-shaped. This percentage is similar to that seen with dnaBts recG (46%), and is lower than seen with other strains, possibly reflecting a change in the equilibrium between fork blockage, fork regression and replication restart (that leads to re-establishment of the blocked signal), with a longer lived HJ intermediate upstream in the absence of RecG. Upon shift to 42°C the level of forked DNA decreased to ~10% at 15 min and by an hour a further decline to 7% Y-DNA occurred (Fig. 5A and B). The non-linear DNA structures detected in the dnaBts recGruvABC mutant upstream of the block dissipated over time becoming faint after 15 min (~3% as Y-DNA) and almost disappeared by 60 min (Fig. 5C; Fig. S5). The signal intensity seen in this region more closely resembled that of dnaBts recG and dnaBts ruvABC rather than maintaining the stronger signals of dnaBts recQrecG and dnaBts recQruvABC.

Fig. 6. Replication restart is impaired in a dnaBts recG mutant and slightly impaired in dnaBts ruvABC and dnaBts recQ mutants. Subsequent to an initial growth at 30°C with ara, a subset of cells were treated with AT for 10 min to release the block (+ara +AT 30°C). The remaining cells underwent a temperature shift from the 30°C to 42°C then returned to 30°C (TS) and exposed to AT for 10 min (+ara +AT TS). Replication intermediate structures visualized by 2-D gel analysis in the array and upstream regions 10 min after addition of AT (A), cell viability (B) and foci percentages (C) under indicated conditions are shown. See also Fig. S6 and Fig. S7.

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Both RecG and RuvABC have several reported functions, some of which overlap. The 2-D gel profiles of the double recGruvABC mutant closely resembled that of each single mutant, suggesting that they did not have an additive effect in inhibiting fork processing, and may be working in the same pathway.

Finally, the effect of the absence of all three proteins together was analysed via a dnaBts recGrecQruvABC mutant. Based on the previous trends, it was expected that the triple deletion mutant should resemble either double mutant containing recQ. Indeed, 60% of the initial Y-shaped DNA signal remained after 60 min at 42°C (Fig. 5). Additionally, the signal patterns detected upstream over the hour of fork collapse in the triple mutant were consistent with the single and double mutants, where the non-linear DNA signals were strong at 0 and 15 min but reduced by an hour (Fig. 5, Fig. S5). This again suggested that RecQ is the major factor controlling fork processing.

**Restart of replication is impaired in a recG mutant**

It has been previously established that the TetR-YFP replication roadblock can be relieved with the addition of AT, resulting in the restart of replication (Fig. 1). The addition of AT for 10 min following 2 h of replication roadblock restored viability to levels seen in isogenic cells that never had arabinose to induce replication stalling. 2-D gel analysis of this replication resumption in dnaBts saw the disappearance of forked DNA (Fig. 6), leaving solely linear DNA, in both the upstream and array regions after 10 min AT exposure, which correlates with the restoration of viability (Fig. 1). The absence of forked DNA meant that all the intermediates previously seen (Fig. 2) had been processed within this 10 min period. The most likely explanation is that replication restart had occurred across the population of cells and the Y-shaped DNA had now been fully replicated. Importantly, replication restart was seen to be efficient even after temperature shift of the dnaBts strain; once the cells had undergone the temperature shift to 42°C for 1 h and then back to 30°C to allow re-establishment of the replisome, only linear DNA resided in the 2-D gels after 10 min of AT exposure (Fig. 6A) (compare to signals in Fig. 2 where AT was not added). As previously noted (Fig. 1) the counting of cell numbers with multiple foci for dnaBts revealed that there was a slower release of the block under these conditions, however, this delay wasn’t seen in the 2-D gels. This suggests that the replication forks have restarted within 10 min and the array has been copied, but there has not been sufficient time for the two daughter copies to segregate from each other.

The dnaBts recG strain produced extremely prominent HJ and Y-shaped signals upstream of the replication roadblock (Fig. 4C; Fig. S5). After addition of AT for 10 min, it was seen that substantial levels of Y-shaped DNA and HJ were still present, both in samples kept at 30°C and after temperature shift to 42°C (1 h) and back to 30°C, in both the array and upstream regions (Fig. 6A; Fig. S6). However, the former spot on the Y-arc, indicative of replication blockage, is now absent showing successful release of the protein roadblock. The absence of RecG left a substantial proportion of HJs and forks (~35% and ~8% was non-linear DNA at the array at 30°C and after TS, respectively) that were yet to be processed and/or migrated in order to restart replication in a timely manner (Fig. S6). Despite the inability of the cells to resolve these intermediates within the 10 min window, they were largely able to eventually recover their viability upon release of the block (Fig. 6B), indicating that this was a delay rather than a failure to restart DNA replication. To ensure the DNA structures visible in the 2-D gels were not an artefact of the presence of DnaBts, the same assay was performed on a recG strain with WT DnaB (Fig. S7). The resulting 2-D gel showed similar signals as for the dnaBts equivalent (Fig. S7).

The addition of AT to the dnaBts ruvABC mutant resulted in restart of replication of the forks at the site of the block, but faint HJ and Y-arc signals remained upstream of the array, both at 30°C and after temperature shifting. In the array region, the HJ and Y-arc signal were faint, but clearly present, though at a lower level than in the equivalent recG mutant (Fig. 6A). This also suggests a slight delay in replication restart, and longer persistence of HJ intermediates in the absence of RuvABC. Overall, the majority of HJs were able to be processed or resolved in the absence of RuvABC (compare Figs 4–6). Following addition of AT to allow replication to proceed through the array, the number of foci within each cell was similar to that seen in dnaBts; 72% of cells had two or more foci at 30°C but following the temperature shift to 42°C only 21% showed multiple foci in the presence of AT (Fig. 6C). Cell viability was also completely restored by 10 min treatment with AT (Fig. 6B).

Linear DNA was the only DNA structure visualized by 2-D gels in both the array and upstream regions of a dnaBts recQ mutant following the release of the roadblock via AT addition at 30°C (Fig. 6A), suggesting complete replication restart. Replication restart was also seen to be similar to dnaBts in terms of cell viability and the number of foci observed per cell following addition of AT (Fig. 6B and C). However, following the temperature shift, lingering intermediate signals remained in both regions (Fig. 6A). Cell viability was seen to be fully restored in the dnaBts recQ following addition of AT (Fig. 6B) suggesting replication restart does eventually occur across the population. Furthermore, the double and triple mutants did not affect replication restart, as their viabilities were shown to be
restored to levels not significantly different from untreated (Fig. S8). Therefore, each mutant strain was able to restart replication in the majority of cells, but there was a distinct delay in the processing of HJs in a recG mutant.

Discussion

It has been proposed that almost all replication forks in E. coli will encounter DNA damage on the template during replication in normal growth conditions (Cox et al., 2000), and that collision with nucleoprotein complexes provides an even greater impediment to replication progression than DNA damage (Gupta et al., 2013). This study examined the relative contributions of RecG, RuvABC and RecQ in the reversal and processing of replication forks that had collapsed at a nucleoprotein block, in vivo. An array of repressor–operator complexes allowed replication to be arrested at a specific location on the chromosome. Processing of the stalled replication fork DNA can be seen by the loss of Y-shaped DNA at the block upon replication fork collapse, which was synchronized by use of the dnaBts allele, concurrent with HJ being seen in the region upstream of the array. The simplest conclusion is that collapse of the replisome leads to RFR, the first processing event thought to occur at a stalled fork. RecG and RuvAB, two branched-DNA specific motor proteins, have previously been implicated in the reversal of stalled replication forks (Seigneur et al., 1998; McGlynn and Lloyd, 2000; Baharoglu et al., 2008; Lemasson et al., 2008). In this study, both dnaBts recG and dnaBts ruvABC mutants showed a similar minor deficiency in fork processing upon replisome dissociation. Thus, both RecG and RuvABC assist in RFR of a blocked fork. However, a greater percentage of forks were found to remain at the block in the recQ mutant highlighting the key role of RecQ in facilitating RFR. Previous studies examining RFR at a transcription/replication collision showed that RFR occurred readily and it was not dependent upon RecA, RecG or RuvAB (De Septenville et al., 2012). This is consistent with the major role of RecQ in RFR we have identified here.

Previously, RecQ has been shown to bind and unwind a diverse range of DNA substrates in vitro (Harmon and Kowalczewski, 1998), including unwinding of dsDNA without a ssDNA gap (Rad et al., 2015), and displays a 3'-5' movement along one strand. This in vitro mechanism of action for RecQ is consistent with the in vivo evidence presented here of RecQ’s involvement in RFR. At a stalled fork, RecQ could load onto the lagging strand gap and act to enlarge the ssDNA region. RecA may then mediate strand exchange to re-pair the two template DNA strands, leading to fork reversal (Fig. 7). Alternatively, RecQ may play another, not mutually exclusive, role at a fork; two RecQ proteins proceeding in opposite directions from the branch point of the fork along the lagging strand template (unpairing the nascent lagging strand) and the nascent leading strand (unpairing it from the leading strand template) could directly facilitate RFR by converting both nascent strands into ssDNA allowing their mutual pairing. Consistent with this, RecQ has been shown in vitro to be able to convert a three-way DNA structure into a HJ, although it does this infrequently (Bagchi et al., 2018).

The key role for RecQ found in this study reflects evidence on replication fork processing in eukaryotes. The human RecQ homologs BLM, WRN and RECQ5 have been implicated in regressing replication forks (Mohaghegh et al., 2001; Kanagaraj et al., 2006; Machwe, Xiao et al., 2006; Ralf et al., 2006; Machwe et al., 2007), and their absence leads to a predisposition to cancer, genetic instability and premature aging. In yeast the Sgs1 protein is the single RecQ homologue and has been shown to associate with stalled replication forks following HU-treatment (Cobb et al., 2003). Thus, the proposed role of RecQ in RFR in E. coli may be functionally conserved between bacteria and eukaryotes.

RecG has been shown in vitro to be able to regress Y-shaped forked DNA into a HJ, but it has a preference for forks where the leading strand is absent or there is a gap on the leading strand (McGlynn and Lloyd, 2001b). However, it would seem likely that a replisome blocked by a protein on the DNA template would synthesize DNA on the leading strand at least as far as on the lagging strand, or more likely even further, as has been seen for replication up to the Tus/ter replication block (Hill and Marians, 1990; Mohanty et al., 1998). Furthermore, if another protein were present blocking the replication fork then it seems likely that this would sterically hinder access of RecG to the duplex DNA it requires for binding, preventing it from catalysing the initial fork reversal. The crystal structure of RecG on a model forked DNA structure showed that it binds to between 10 and 25 bp of duplex DNA ahead of the branch point (Singleton et al., 2001). Together, the structural preference of RecG and the steric hindrance from a protein roadblock may explain why RecG appeared to play a relatively minor role in RFR following encounter of the replisome with a protein roadblock. RecG may be more active in RFR on other substrates.

RuvAB has been implicated in RFR in certain replication mutant backgrounds, such as following inactivation of the dnaEts allele (Baharoglu et al., 2006). RuvAB has also been shown to be able to regress a fork in vitro to form a HJ, but it does so with low efficiency, preferring to unwind DNA in the opposite direction from that required to form a HJ (McGlynn and Lloyd, 2001a). Conversely, RuvAB has been shown to efficiently migrates a pre-formed HJ.
RecQ promotes replication fork reversal

Fig. 7. Model for replication fork reversal at a protein roadblock. Template DNA is shown as black lines and newly replicated strands are in blue. A proteinaceous roadblock to replication is shown as a grey oval. The most likely disposition of the leading and lagging strands is shown, with the leading strand coming close to the site of the block. Upon this substrate RecQ can act, possibly in concert with RecJ, to lengthen the ssDNA gap on the lagging strand template. RecFOR could then load RecA on the ssDNA, leading to strand exchange which pairs the two template strands, displacing the nascent leading strand. The initially regressed fork can then be acted on by a number of pathways such as the known branch migration proteins RecG and RuvAB which could move the HJ upstream. RecG may also reverse this process to re-create the Y-shaped DNA to allow replisome reloading. Nuclease action by Exol/SbcCD, RecQ/J and/or RecBCD could also remove the two nascent DNA strands to eventually re-generate a Y-shaped fork upstream from the site of the roadblock. [Colour figure can be viewed at wileyonlinelibrary.com]
in vitro (Parsons et al., 1995). It has been proposed that RuvAB migrates HJs that result from RFR by other proteins, with RecG having been the preferred candidate to act at the stalled fork (Buss et al., 2008). Consistent with this, a ruvABC deletion showed only a mild defect in direct RFR in this study. The similarity between the phenotypes of ruvABC, recG and the combination recGruvABC double mutant in these assays supports the notion that they may play roles in the same pathway. It is likely that the pathway utilized for processing a stalled fork is dependent upon the exact structure of the DNA at the branch point, namely the relative disposition of the leading and lagging strands. A heterogeneous population of DNA structures in vivo may account for the fact that a proportion of forks are processed in the absence of RecQ. In addition, multiple overlapping pathways may exist to catalyse RFR and in the absence of RecQ a less efficient pathway predominates. The diversity in the possible structures of the DNA at a replication block may account for differences seen in processing pathways utilized by the cell, particularly after treatment with UV where previously a RecF-pathway dependent processing event has been found to repair the fork (Jeiranian et al., 2013).

When mutations in recQ were combined with recG and ruvABC, it was found that there was a further decrease in the efficiency of RFR with ~60% and ~55% of replication forks, respectively, remaining unprocessed after 1 h following inactivation of DnaBts. The recQrecGruvABC mutant showed a similar decreased efficiency of RFR to the double mutants, with ~60% of the replication forks remaining after 1 h at 42°C. This result again suggests that RecG and RuvABC may be associated in their own processing pathway. Concurrently with this there was an increased HJ signal upstream of the block in the double mutants, suggesting a delay in dealing with processing the HJs that result from the reduced level of RFR.

RFR is only one possible mechanism to deal with a collapsed replication fork; another proposal is that direct endonuclease action on an arrested fork can lead to breakage of one arm at the fork, which can then be repaired by homologous recombination. However, the results presented here show that this did not occur at the majority of forks. Stalled Y-shaped DNA in a recQ mutant was largely stable over an hour suggesting nuclease action must be slow or only works on a sub-population of fork structures, with the majority normally being processed by RFR. It is most likely that the HJ observed in the DNA immediately upstream of the replication roadblock is the direct result of RFR, rather than being an intermediate in repair of a free double stranded end by homologous recombination. Indeed, mechanistically it makes sense for the cell to avoid producing a broken DNA molecule if possible, as this can lead to potentially mutagenic repair processes.

The other possible processing event at the collapsed replication fork is the action of exonucleases that digest the two nascent DNA strands, an activity that has been previously reported when DnaBts is inactivated and is attributed to RecJ and Exol (Belle et al., 2007). There is indeed a Y-arc signal seen in the upstream DNA, the result of exonuclease action. However, what is unclear is whether the exonucleases act on the DNA of the Y-shaped fork, or on the ends of the reversed HJ produced by RFR, as these would both produce the same endpoint structure in the upstream DNA. Quantification of these signals showed that there is less DNA in the Y-arc upstream than is seen to be in the HJ form. It should also be noted that the HJs detected are most likely an underestimate of the HJ levels in vivo, as the HJs are not trapped or crosslinked. Each HJ is free to branch migrate due to the homology throughout its sequence. Branch migration all the way to one end of a DNA arm will dissociate the HJ and dissolve into two linear DNA duplexes. This process would be essentially irreversible. Cell lysis, washing and DNA digestion occur over the period of days giving ample time for some HJ dissolution. Similarly, if one arm of the HJ has been partially digested by exonucleases, then branch migration to the end of this arm would convert the HJ to Y-shaped DNA. Given these caveats about the observed level of HJ seen, then it seems plausible that RFR is the major pathway utilized in cells and the HJ level observed underestimates this.

The SOS regulatory response is induced by the prolonged presence of RecA filaments on ssDNA. No evidence for SOS induction was detected 2-h after induction of a replication roadblock (Fig. 3). During the experiments described here the typical fork-blocking induction period is 90 min, which is not long enough to induce SOS. This is then followed by an additional incubation at either 42°C or 30°C, for up to 1 h. It is possible that prolonged incubation of the dnaBts strain at 42°C will induce SOS, but certainly the initial processing events should be independent of the SOS response. Further, the processing events observed in the dnaBts strain were similar to those seen in WT, with the dnaBts allele used simply as a means to amplify processing events and the majority of RFR occurring in the first 15 min (Fig. S3). Thus, it is likely that the RFR visualized in a dnaBts strain remains a valid interpretation of the mutants’ results and not an artifact of SOS response processing within the timeframe assessed. The release of a replication fork block by addition of AT did not lead to activation of the SOS response suggesting that replication can recover quickly once TetR-YFP leaves the DNA.

Although the effect of deletion of recG upon RFR was modest, it was clear that in the absence of RecG a much stronger HJ signal was seen in the region upstream of the replication roadblock. This implicates RecG in the timely processing of HJs. The simplest and least recombinogenic
mechanism to deal with a HJ produced by RFR is to branch migrate the junction back into a Y-shaped fork, upon which the replisome can be reloaded, and this may be a role played by RecG. recG mutants also showed an accumulation of HJ structures during replication restart (Fig. 6, Fig. S6 and S7) suggesting that HJs persist even after the replication roadblock has been removed in these cells, although the viability data suggests that the delay to recovery is not fatal and is eventually overcome. The deletion of ruvABC was also seen to increase the level and persistence of HJs in the upstream DNA region, consistent with the known role of the complex in branch migration and resolution of HJs.

It is noteworthy that HJs are seen to be present upstream of the replication roadblock in both recGruvABC and recQrecGruvABC backgrounds, and that the levels of these intermediates decline over time, along with a reduction in Y-shaped DNA at the block. There is clearly another process able to either migrate the HJs out of the region being probed or to resolve the HJ. As a result of this finding, we concluded that neither RecG nor RuvABC are absolutely required for HJs from RFR to be processed. RusA, the only other known endogenous HJ resolvase enzyme in E. coli, is absent from the strains used in this study. Spontaneous branch migration of the HJ could explain these results, either leading to restoration of the fork or movement out of the region examined, but only the former would allow for replication restart. Alternatively exonucleases could digest away one arm of the HJ to restore a Y-shaped fork for replisome reloading. Exol or SbcCD can digest a 3’ DNA overhang (Connelly et al., 1999; Long et al., 2010), whilst RecJ can digest a 5’ ended strand. Once the DNA end is blunt, RecBCD can load and processively digest both strands.

The proposed model (Fig. 7) has some testable predictions. The establishment of the system described here to study replication fork processing in vivo can be built upon in future work to examine the roles of other proteins such as RecA, RecFOR, SbcCD, RecBCD, XonA and RecJ. This could lead to a comprehensive understanding of the complex and overlapping roles played by the many homologous recombination proteins in this key DNA repair process.

**Experimental procedures**

**Strains and plasmids**

Bacterial strains were derivatives of E. coli K12 AB1157 (Bachmann, 1972) which carry 240 copies of tetO in an array within the lacZ gene (Wang et al., 2006) and the pKM1 plasmid encoding the TetR-YFP repressor under the control of the Para promotor (Metrick and Grainge, 2016). Lambda Red recombination was conducted initially to replace recQ, recG, ruvABC, recA and uvrD with a spectinomycin resistance cassette (Datsenko and Wanner, 2000). The resistance cassette was subsequently removed by Flp recombinase, leaving only an FRT site flanked by the start and stop codons (Datsenko and Wanner, 2000). The temperature-sensitive dnaBts allele (Carl, 1970) and all gene knockout strains were created by P1 transduction and confirmed by PCR (Table S1 for list of strains). The sulAp-mCherry construct, created by fusing the sulA promoter to mCherry, was inserted into the chromosome approximately halfway between ori and ter on the left replichore by Lambda Red recombination in a non-coding region while the native sulA gene remained.

**Bacterial growth**

Cultures grown overnight at 30°C in L-broth were diluted to OD₆₀₀nm = 0.01 in a dilute complex medium (0.1% tryptone, 0.05% yeast extract, 0.1% NaCl, 0.17 M KH₂PO₄, 0.72 M K₂HPO₄). Cells were supplemented with ampicillin (100 μg ml⁻¹) and/or kanamycin (50 μg ml⁻¹) as required. Production of the fluorescent repressor TetR-YFP was induced by addition of 0.1% (w/v) arabinose once cells had reached at least OD₆₀₀nm = 0.05. Cells were then incubated for 90 min and examined under a fluorescence microscope as described previously (Metrick et al., 2016) to confirm the extent of replication blockage across the cell population. A minimum of 200 cells were assessed and foci counted. Cells were shifted to 42°C for an hour to induce replisome collapse in the temperature sensitive dnaBts strain. Subsequently, the cells were shifted back to 30°C for 10 min. Tight repressor binding was relieved by the addition of the gratuitous inducer anhydrotetracycline (AT; 10 μg ml⁻¹). Cell viability was determined by performing a 10-fold serial dilution and 5 μl of each dilution was spotted on agar containing the required antibiotic (and AT if needed). Selected dilutions were spread onto agar to determine CFU per milliliters. All plates were grown at 30°C overnight.

For sulAp-mCherry strains, an overnight culture was diluted in complex medium and grown to at least OD₆₀₀nm = 0.05 at 30°C before 0.1% arabinose was added. Samples were taken after 2, 4, 6 and 24 h. After the two hours incubation, a subculture was treated with AT (10 μg ml⁻¹) and samples were taken after 2, 4 and 22 h of growth at 30°C. Samples were re-suspended in PBS and Relative Fluorescence Units (RFU) of mCherry were detected with the FLUOstar micro-plate reader (BMG Labtech). The final RFU value was determined by normalizing to cell density (OD₆₀₀nm) then subtracting the normalized RFU of the untreated control from the same time point.

**2-D DNA agarose gel electrophoresis and Southern hybridization**

Samples of cells were taken at the indicated time points and the DNA prepared as previously outlined (Metrick and Grainge, 2016; Metrick et al., 2016). DNA was digested with either EcoRI (array region) or HindIII (region upstream of the array). 2-D gel conditions and Southern hybridization were as described by (Metrick et al., 2016). Statistical analysis was determined using the Student's t test. Quantification
of 2D-gel signals was conducted in MetaMorph® and ImageQuant TL softwares.

Author contributions

Conceptualization, K.M. and I.G.; Methodology, I.G., and K.M.; Investigation, G.W., K.M., T.C. and A.G., Writing – G.W. K.M. and I.G.; Review & Editing, G.W., K.M., A.G. and I.G.; Funding Acquisition, I.G.; Supervision, K.M. and I.G.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.