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Termination of DNA replication at Tus-ter barriers results in under-replication of template DNA

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Katie H. Jameson¹ , Christian J. Rudolph², and Michelle Hawkins^{1,*} 

From the ¹Department of Biology, University of York, York, UK; ²Division of Biosciences, College of Health, Medicine and Life Sciences, Brunel University London, Uxbridge, UK

Edited by Patrick Sung

The complete and accurate duplication of genomic information is vital to maintain genome stability in all domains of life. In *Escherichia coli*, replication termination, the final stage of the duplication process, is confined to the “replication fork trap” region by multiple unidirectional fork barriers formed by the binding of Tus protein to genomic *ter* sites. Termination typically occurs away from Tus-*ter* complexes, but they become part of the fork fusion process when a delay to one replisome allows the second replisome to travel more than halfway around the chromosome. In this instance, replisome progression is blocked at the nonpermissive interface of the Tus-*ter* complex, termination then occurs when a converging replisome meets the permissive interface. To investigate the consequences of replication fork fusion at Tus-*ter* complexes, we established a plasmid-based replication system where we could mimic the termination process at Tus-*ter* complexes *in vitro*. We developed a termination mapping assay to measure leading strand replication fork progression and demonstrate that the DNA template is under-replicated by 15 to 24 bases when replication forks fuse at Tus-*ter* complexes. This gap could not be closed by the addition of lagging strand processing enzymes or by the inclusion of several helicases that promote DNA replication. Our results indicate that accurate fork fusion at Tus-*ter* barriers requires further enzymatic processing, highlighting large gaps that still exist in our understanding of the final stages of chromosome duplication and the evolutionary advantage of having a replication fork trap.

In all domains of life, DNA replication is initiated at origins which direct the assembly of multisubunit replication complexes called “replisomes.” Once fully assembled, replisomes travel along the DNA as replication fork complexes, which move away from each other in opposite directions. They progress until they reach a chromosome end or converge with a replication fork complex traveling in the opposite direction resulting in a fusion event (1). The number of fusion events is directly correlated to the number of origins in each organism. This means that the number of expected fork fusions varies significantly between organisms; from thousands in metazoa to a few in some archaea and only one in most bacteria (2).

The ability to carry out the convergence and fusion of replication fork complexes with high accuracy is essential to the maintenance of genomic stability and cell survival (3). Recent studies in both prokaryotes and eukaryotes have highlighted the fact that the fusion of replication fork complexes requires careful orchestration by a variety of protein cofactors (1, 3–7).

Like the majority of bacteria, *Escherichia coli* has a single circular chromosome which is replicated bidirectionally from a single replication origin (*oriC*). The single origin dictates that each chromosomal half or “replichore” is duplicated by a single replisome and the number of replication fork fusions is restricted to exactly one, which takes place midway around the chromosome (8) (Fig. 1A). The location of termination is constrained by a specialized replication fork trap formed by a series of polar blocks which allow replisomes to enter but not leave the termination region. These polar blocks are created by asymmetric binding of the Tus terminator protein to a series of 23 bp nonpalindromic *ter* sequences (*terA–f*) distributed at either side of the termination region (9, 10) (Fig. 1, A and B). Five *ter* sites flank both sides of the termination region. Each replisome is able to bypass the first five sites it encounters in the permissive direction by displacing Tus protein. However, the replisome will be arrested at any Tus-*ter* complexes encountered in the nonpermissive orientation (6, 11).

The asymmetry of replisome arrest at the Tus-*ter* complex has been extensively studied (5, 11). These studies have demonstrated that polar arrest at Tus is triggered by the approaching replisome unwinding the double-stranded DNA immediately adjacent to the nonpermissive face (12). This induces specific DNA-Tus contacts which generate a “locked” complex that cannot be bypassed by the oncoming replication machinery. Specifically, a base on the leading strand template (C6) flips into a specialized binding site on Tus (Fig. 1B), guided by nearby residues, to create a sustained barrier (12, 13). Unwinding alone has been shown to be sufficient to generate a “locked” Tus-*ter* complex (13, 14). Nevertheless, the block may be further stabilized by an interaction between Tus and the replicative helicase, DnaB, which is thought to sit at the head of the replisome (15).

Although the trap system limits replication fork fusions to the termination region, fusions typically occur away from Tus-

* For correspondence: Michelle Hawkins, michelle.hawkins@york.ac.uk.

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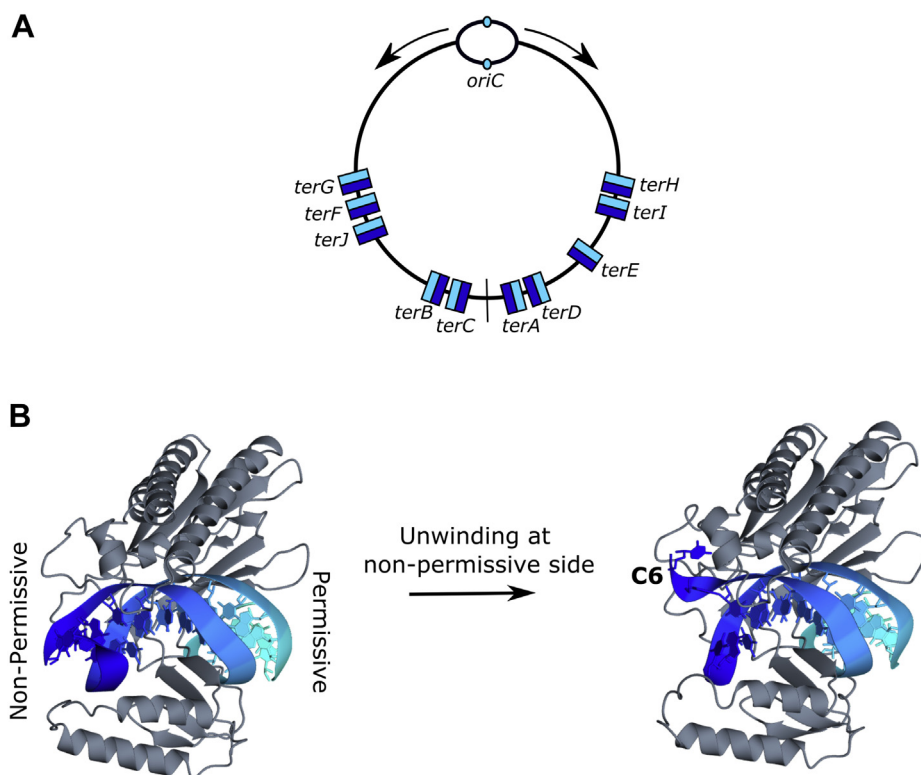


Figure 1. DNA replication and Tus-ter termination trap in *E. coli*. A, *E. coli* contains a single circular chromosome, which replicates bidirectionally from a single origin (*small oval*). Direction of replisome travel from the origin is depicted by *arrows*. Chromosomal midpoint indicated by a *straight line*. Location of *ter* sites on the *E. coli* chromosome is shown relative to *oriC*. Permissive orientation displayed in *light blue*, nonpermissive orientation displayed in *dark blue*. B, structure of Tus-ter (PDB ID: 2l06) illustrating the nonpermissive and permissive faces (*left*) and the “locked” conformation formed by DNA unwinding at the nonpermissive face (*right*). The cytosine base at position 6 of *ter* (C6), which flips into a specific binding site on the nonpermissive face of Tus to form the “lock,” is indicated.

ter sites in the majority of cells. This was demonstrated by early labeling experiments (16), and more recently, marker frequency analysis produced by Deep Sequencing has shown that fusion typically occurs close to the numerical midpoint of the chromosome (17). Deletion of *tus* from wild-type cells results in only mild distortion of nucleic acid metabolism and does not significantly change the location of replication fork fusion, demonstrating that it is not a requirement for replication termination (18, 19). Recent analysis has illustrated that replication fork traps are not widely distributed among bacteria (20). However, fork traps are found not only in some Gram-negative bacteria such as *E. coli*, but also in the Gram-positive *Bacillus subtilis*. These two fork trap systems show no significant sequence or structural similarity, indicating that they have evolved *via* convergent evolution (11). This indeed suggests that the fork trap conducts an important physiological function.

Multiple functions of the fork trap have been proposed. One early suggestion was the potential to contain over-replication of the genome (21). This theory has been bolstered in recent years by the observation of over-replication of the terminus region in the absence of RecG (17) and 3'-exonucleases (22, 23). These enzymes are thought to be involved in the processing of intermediates that form as a result of fork fusion events and can lead to replication restart if the intermediates persist for longer than normal (8, 23). This model is supported by the fact that

over-replication is either eradicated or significantly reduced in cells lacking either RecG or 3'-exonucleases following linearization of the chromosome in the termination area, which will prevent replisomes from fusing (17, 23, 24). Additionally, over-replication in the absence of RecG can be induced in ectopic chromosomal locations if replisomes are forced to fuse in these ectopic locations (25). These observations are in line with the idea that intermediates formed as part of termination can trigger replication restart and lead to over-replication of the chromosome (17, 23). Thus, one important role of the fork trap might be to block forks initiated within the termination area traveling toward the origin.

Another scenario where the replication fork trap will come into play is when one replisome is stalled before it reaches the chromosomal midpoint. Studies with ectopic chromosomal origins suggest that Tus-ter is an effective barrier *in vivo* when one replisome is delayed, and it has been shown that a subset of fusion events occurs at Tus-ter barriers *in vivo* (26). In this scenario, fusion must occur at Tus-ter when a replisome paused at the nonpermissive face of Tus is met by a moving replication fork complex coming toward the permissive face. Surprisingly little is known about what happens in this scenario as biochemical studies of Tus-ter have typically focused on understanding what happens when a replisome approaches Tus-ter from either the blocking or permissive orientation but not when replisomes meet at Tus-ter.

A recent *in vitro* study found that the addition of Tus-ter to a plasmid replication system at a location mimicking that of the normal chromosomal arrangement inhibited the formation of circular monomers (27). This inhibitory effect could be overcome by increasing the spacing between Tus-ter such that it was likely not to be the fusion point of the replication forks; or by adding UvrD (27), an accessory helicase reported to overcome Tus-ter barriers (28). This result suggests that additional processing steps are required for the fusion of a replisome paused at Tus-ter with a freely moving replication fork complex.

We have designed an *in vitro* DNA replication system where we can control the fusion of two replication forks at a Tus-ter complex and monitor how far each replisome progresses during replication fork fusion. Our data demonstrate that the fusion of a fork paused at a Tus-ter complex with a freely moving fork will not result in fully replicated templates. This fusion scenario leaves a gap of 15 to 24 bp of DNA between the two replisomes that remains unreplicated. These results raise further questions about how fork fusion events are completed at Tus-ter *in vivo* and renew the debate about the physiological advantage of having a replication fork trap.

Results

Controlled fusion of replisomes at Tus-ter

We used an *in vitro* plasmid replication system to study what happens when a moving replication fork complex fuses with another replication fork complex paused at Tus-ter. In the presence of DNA gyrase and reconstituted *E. coli* replication machinery, bidirectional DNA replication can be initiated from an *E. coli* *oriC* sequence on a plasmid template *in vitro* (29). Replisomes are able to progress around the majority of the template but eventually stall due to positive supercoiling, leaving the last ~150 bp of DNA unreplicated. This tension can be released by linearization of the plasmid template with a restriction enzyme, thereby allowing replication of the last 150 bp of DNA (29). Here, we initiated replication from the *oriC*-containing plasmid, pKJ1, which can be linearized by the addition of SmaI (Fig. 2A). Unimpeded, the major product of this reaction is a 6 kb band, which corresponds to full-length leading strand replication products from one of the two replisomes progressing around the entire template before the release of the other (Fig. 2B, lane 1). The processive nature of the replisome means that *E. coli* can replicate DNA at approximately 600 to 800 bp/s (30, 31), thus if there is a >10 s delay in the release of one replisome from *oriC*, the other replisome is able to complete replication before the second replisome has been released. A smear of products is also observed at <6 kb corresponding to leading strand products from replisomes meeting stochastically throughout the plasmid. Lagging strand products are observed as a smear of products centered around 0.5 kb (Fig. 2B, lane 1). Replisome progression can be blocked by the use of protein binding sites on the template DNA. We have taken advantage of two such blocks to generate a system where we are able to block

replisomes traveling in both directions, before releasing one to allow fusion to occur in a controlled manner.

Replisomes can be blocked at a *lacO*₂₂ array bound by LacI and subsequently released by the addition of IPTG (32), enabling them to continue replication (Fig. 2C). Replisomes can also be blocked at Tus bound *ter* sequences if the replisome encounters Tus-ter in the nonpermissive direction (Fig. 2D). Replisomes that encounter Tus-ter in the permissive direction are able to displace Tus and travel all the way round the template (33) (Fig. 2D). Unlike LacI-*lacO* blocks, replisomes blocked at Tus-ter cannot be released. We have used both blocks to create a system where we can control the fusion of two replication forks directly at a Tus-ter complex. We initiate bidirectional replication on pKJ1, block replisomes traveling in the clockwise direction at Tus-ter_B, and those traveling in the counterclockwise direction at *lacO*₂₂ (Fig. 2E); this generates leading strand products of 2.7 kb and 1.5 kb, respectively (Fig. 2B, lane 5). We then release the replisome blocked at *lacO*₂₂ by addition of IPTG, allowing it to resume synthesis and consequently meet the blocked replisome at Tus-ter_B, yielding 2.7 kb and 3.3 kb products (Fig. 2B, lane 6 and Fig. 2E). A small quantity of full-length product (6 kb) is also always observed in these reactions. We reason that this is likely due to a small percentage of template molecules, which have replisomes traveling unidirectionally counterclockwise and hit Tus-ter_B from the permissive side (Fig. 2D). We anticipate a similar number of unidirectional replisomes traveling in the clockwise direction, but these cannot be quantified because they contribute to the 2.7 kb products.

We confirmed that both blocks work independently of one another using the same template and reaction conditions. As expected, replisomes blocked at a *lacO*₂₂ array bound to LacI generate products of 3.7 kb (clockwise) and 1.5 kb (counterclockwise) (Fig. 2B, lane 2 and Fig. 2C). Release by the addition of IPTG produces a smear of products from replisomes meeting within the *lacO* array (3.7–4.1 kb) and (1.5–1.9 kb) and full-length (6 kb) products (Fig. 2B, lane 3). The addition of Tus alone produces a mixture of products from replisomes, which are blocked and meet at Tus-ter_B (2.7 kb, clockwise, 3.3 kb, counterclockwise), and 6.0 kb full-length products, which first reach Tus-ter_B from the permissive side (counterclockwise) (Fig. 2B, lane 4 and Fig. 1D).

Replisomes fusing at Tus-ter leave a 15 to 24 bp gap

Using the replication assay described above, we analyzed the length of the leading strand products from each replisome to understand how far each replisome can travel during fusion at Tus-ter_B. In an assay we call “termination mapping,” we introduced unique single-strand nicking sites approximately 100 bp upstream of *terB* on each strand of the replication template and used these to cut out individual leading strands from the final replication products (Fig. 3A). We analyzed the size of these products to determine the stop sites of the leading strand polymerases during the reaction. Nicked products were analyzed by denaturing urea PAGE, with reference to sequencing products from cycle sequencing

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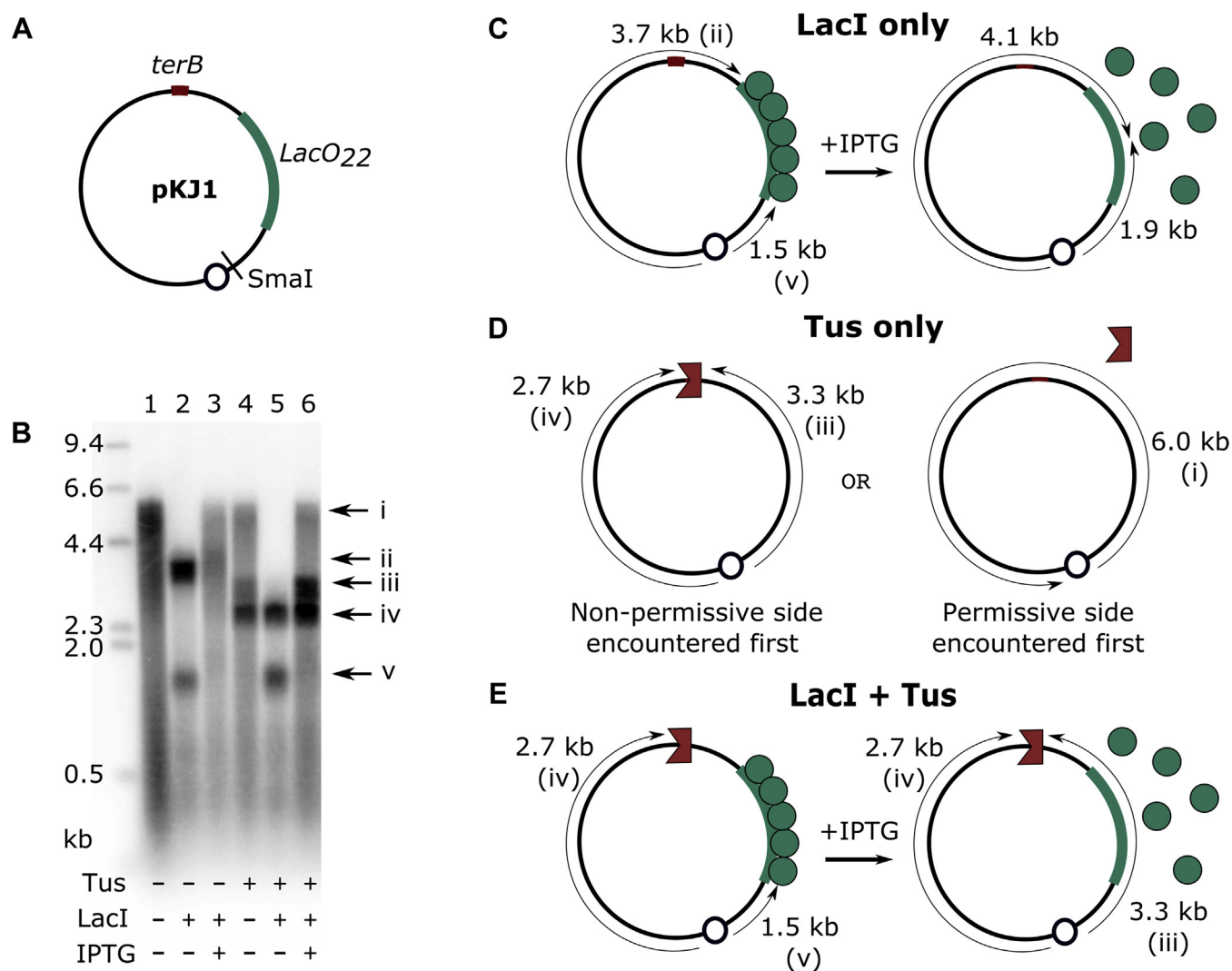


Figure 2. Replication fork fusion can be controlled to occur at Tus-terB. *A*, pKJ1 replication assay template, indicating the location of replication initiation at *oriC* (small circle) and the SmaI cleavage site. The locations of *lacO*₂₂ and *terB* are indicated. *B*, denaturing agarose gel of replication products from pKJ1. Representative gel of >15 replicates. Replication reactions contain 40 mM HEPES pH 8.0, 10 mM Magnesium Acetate, 10 mM DTT, 2 mM ATP, 200 μM GTP/UTP/CTP, 40 μM dNTPs, 0.1 mg/ml BSA, 200 ng template DNA (approx. 2 nM), 50 nM polymerase III core (αεθ), 25 nM clamp loader complex (τ₃δδ'χψ), 160 nM DnaB, 160 nM DnaC, 1 μM SSB, 80 nM β-clamp, 30 nM HU, 200 nM DnaG, and 133 nM Gyrase (A₂B₂) in a final volume of 15 μl. Where indicated, 400 nM LacI, 400 nM Tus, or both proteins are present at the start of the reaction. Reactions are initiated by addition of 300 nM DnaA. After 2 min active replication, 30 units of SmaI (Promega) and 46 kBq [*a*-³²P]-dCTP (111 TBq/mmol) are added. Where indicated, 1 mM IPTG is also added after 2 min active replication. Band labels correspond to replication products shown in panels C–E. *C*, replisome movement in the presence of LacI and its release by the addition of IPTG, with expected leading strand product lengths. *D*, replisome movement in the presence of Tus, including expected leading strand replication products, depending on which side of Tus is encountered first. *E*, replisome movement in the presence of LacI and Tus, before and after release of LacI by addition of IPTG. Expected leading strand replication product lengths are indicated.

reactions carried out using the same template DNA. The DNA-binding location of the 5'-terminus of the primers used for cycle sequencing was identical to the nicking site (Fig. 3A), meaning that comparison of the length of the nicked product with the sequencing reaction products provided a direct readout of the 3'-stop site of DNA replication. Nicked products (N, Fig. 3, B and C) were compared with products from the same reaction that had not been treated by a nicking enzyme (C, Fig. 3, B and C), to ensure there was no misinterpretation of products generated during the replication reaction. Any products observed across both nicked and untreated lanes were ignored. This analysis showed that synthesis of DNA from replisomes traveling clockwise toward

Tus-terB in the nonpermissive direction stopped at an adenosine immediately upstream of *terB* (Fig. 3, B and D). This is in line with previous replisome mapping studies, which found that the main leading strand stop location at the Tus-ter nonpermissive interface was one base closer to *ter* (33, 34). Leading strand synthesis in the opposing direction, traveling toward Tus-terB in the permissive direction, stopped at several locations immediately upstream of and within *terB* (Fig. 3, C and D). These results cannot be attributed to spurious exonuclease activity (from the SSB preparation contaminant, see Experimental procedures and Fig. S1 for details) because there are no 3' ssDNA substrates available during replication and the specific end points we

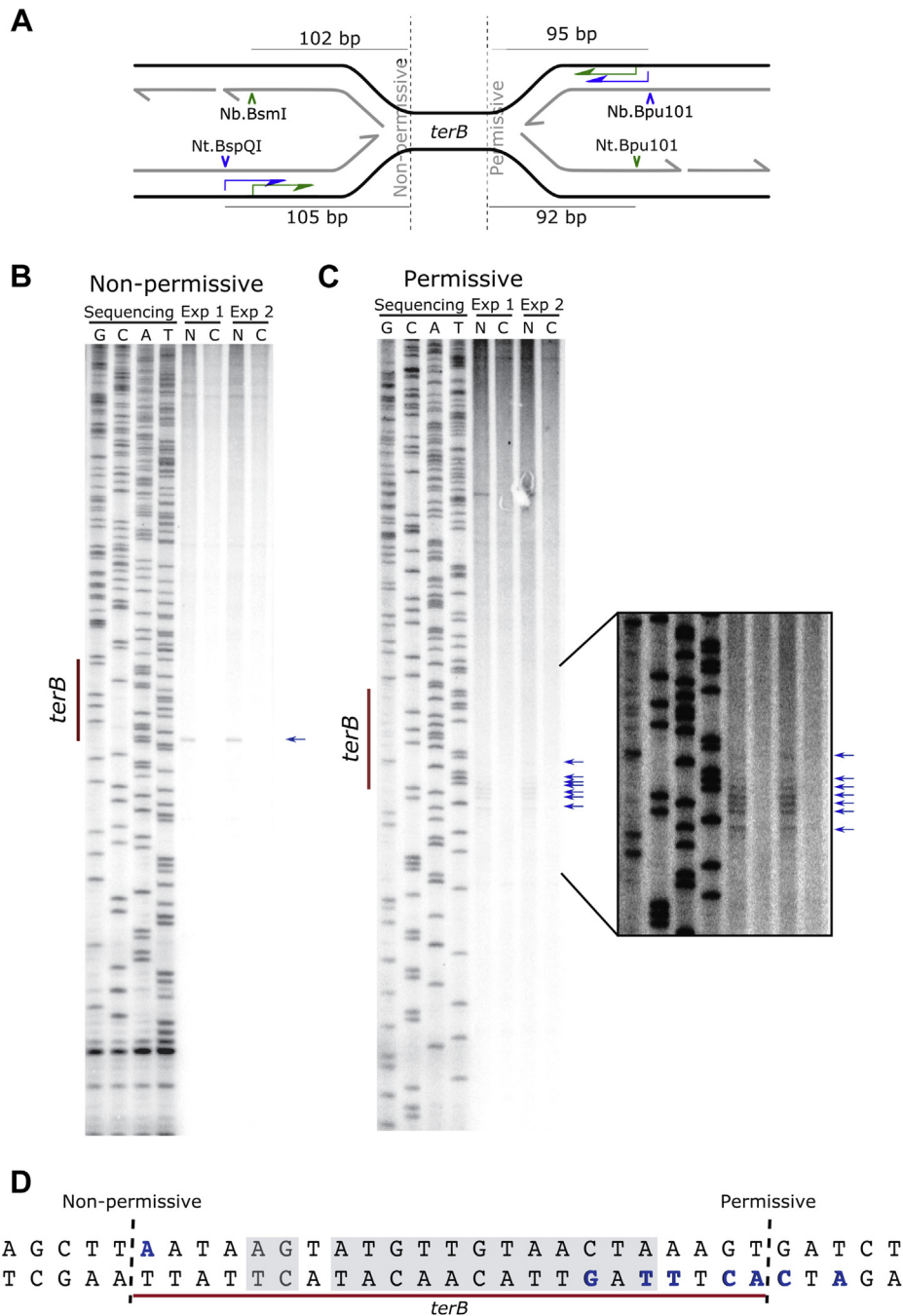


Figure 3. Replication fork complexes meeting at Tus-ter leave a gap of 15 to 24 bp of unreplicated DNA. Analysis of leading strand products created during replisome fusion at Tus-terB. Replication products from controlled fusion reactions at Tus-terB were treated with Nt.BspQI or Nb.Bpu101 to excise leading strand products and determine individual replisome progression during fusion at Tus-terB. A, locations of single strand nicking sites and primer annealing sites for cycle sequencing on pKJ1, with respect to terB. The 5' end of each primer corresponds to a single nicking site, enabling replication product stop sites to be determined. B, mapping analysis of the leading strand product approaching Tus-terB in the nonpermissive direction. Representative gel of three replicates. Nicked products (stop sites) are indicated by arrows. C, mapping analysis of the leading strand product approaching Tus-terB in the permissive direction. Representative gel of three replicates. Nicked products (stop sites) are indicated by arrows. D, terB sequence indicating leading strand stop locations (bold) and the Tus-binding site (shaded area).

report are inconsistent with the variable degradation products expected from exonuclease activity.

We attempted a similar analysis of the lagging strand products, this time introducing a nicking site on the strand complementary to the sequencing products (Fig. 3A). The nicking site corresponded to the 5' end of the primer (but on

the complementary strand). Comparison of nicked and cycle sequencing products would therefore allow us to identify the start sites of lagging strand synthesis. Again, nicking sites were introduced approximately 100 bp upstream and downstream of terB. However, we observed no products specific to our nicking enzyme analysis. Reasoning that we may be too close

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to *terB* to observe a lagging strand start site, we moved the nicking sites approximately 1000 bp away from *terB* and performed a wider analysis. Again, we observed no products that could be clearly identified as specific to the nicking analysis. We attempted to indirectly identify the closest lagging strand start site to *terB* by extending the leading strand replication products in order to determine where they met the lagging strand. Using the Klenow fragment of DNA Polymerase I, we observed that the previously characterized nicked products disappeared when Klenow was added and given time to extend. However, we did not detect any larger products in the extension reactions (data not shown), which suggests that the extension products are variable and too diffuse to measure. We therefore suspect that the lagging strand start sites are too stochastic to be observed by these mapping methods.

Although we cannot map the precise location of the lagging strand start sites nearest to Tus-*terB*, the stop locations of the leading strands provide an insight into how far each replisome is able to travel during fork fusion at Tus-*terB*. Taking both the minimum and maximum distances between the ends of both leading strand products, a gap of 15 to 24 bp of DNA remains unreplicated during fusion at Tus-*terB*.

Unreplicated DNA at Tus-terB is a consequence of fusing replisomes

To confirm that the gap of unreplicated DNA observed in our mapping analysis is a direct consequence of two replisomes fusing at Tus-*terB*, we analyzed what happens when replisomes are limited to travel in only one direction toward Tus-*terB* (i.e., approaching the permissive or nonpermissive face). To control the direction of travel of the replisome through Tus-*terB*, we carried out experiments in the absence of gyrase. In these reactions, only one replisome is able to travel to any extent away from *oriC*, replicating approximately 1 kb of DNA before pausing due to the accumulation of positive supercoils in the DNA. This replisome can be subsequently released by linearization of the template with a restriction enzyme. As long as the restriction enzyme cleaves the template behind the progressing fork, it can then continue replicating the remainder of the DNA template (29). Replisome release is stochastic, meaning an equal number of replisomes are released in either direction (21). When we linearize with SmaI (Fig. 4A, i), a replication fork, which has progressed in either direction, will be released. We have taken advantage of this phenomenon by introducing template linearization sites >1 kb away from *oriC* (Fig. 4A, i); in these reactions, we cut the template in front of one fork, removing the template in one direction. In this way we are able to analyze only products from replisomes traveling toward Tus-*terB* in either a clockwise or counterclockwise direction. Thus, by linearizing the template at NcoI (1.9 kb clockwise), all clockwise (nonpermissive) products can only progress to 1.9 kb in length, and we can analyze what happens when replisomes travel toward Tus-*terB* in the permissive direction without a replisome blocked at the nonpermissive side (Fig. 4A, iii). Conversely, if we cut with SacI (1.6 kb counterclockwise), all

counterclockwise replisomes can only replicate 1.6 kb of DNA, and we can analyze what happens to replisomes approaching Tus-*terB* in the nonpermissive direction (Fig. 4A, ii).

When templates are linearized with SacI (analyzing replisomes traveling clockwise toward Tus-*terB* nonpermissive face), we consistently see the expected 1.6 kb product from counterclockwise traveling replisomes. In the presence of Tus, we see products corresponding to replisomes that have been blocked at Tus-*terB* in the clockwise direction (2.7 kb band, Fig. 4A, ii and Fig. 4B, lanes 10–12), indicating that Tus blocks replisomes in this linearized system.

When templates are linearized with NcoI (analyzing replisomes traveling toward Tus-*terB* permissive face), products are seen which correspond to replisomes traveling through Tus-*terB* in both the presence and absence of Tus (4.1 kb bands, Fig. 4A, iii and Fig. 4B, lanes 13 and 16), and after the release of the counterclockwise replisome from *lacO*₂₂ (4.1 kb band, Fig. 4B, lane 18). These data confirm that the halting of replisomes traveling in the counterclockwise (permissive) direction (3.3 kb band, Fig. 2D, lane 6 and Fig. 4B, lane 6) requires the presence of a replisome at the nonpermissive face on the other side of Tus. This confirms that the unreplicated DNA region seen at Tus-*terB* in our termination mapping assay is a direct consequence of a replisome fusion event at Tus-*terB*.

The replication gap cannot be closed by the lagging strand processing enzymes, RNase HI, DNA Ligase, and DNA Polymerase I

Our standard replication reactions lack RNase HI, DNA Ligase and DNA Polymerase I (called RLP from here onward for brevity), enzymes required for joining lagging strand fragments (and by extension, fusing lagging, and leading strands to one another). Thus, we investigated whether the addition of these enzymes to our replication assay could process the observed gap in replication. We reasoned that closing the gap would result in an increased proportion of full-length replication products compared with all other products from the same reaction; therefore we compared the percentage of full-length replication products in the presence and absence of RLP. Although we consistently observed an increased intensity across all replication products and a small increase in the percentage of full-length products (<10%; Table S1), there was not the substantial increase in the proportion of full-length product we would expect if RLP was able to promote complete termination of DNA replication at Tus-*ter* (Fig. 5A, compare lane 1 with lanes 5 and 6). We noted that we observed a greater increase in full-length products at higher ligase concentrations (+6% at 50 nM versus +3% at 25 nM, Table S1), suggesting that this increase may actually be a consequence of off-target ligase activity within the assay. Note that an additional band at 4.2 kb is observed in reactions containing RLP due to the ligation of 1.5 kb (LacI-blocked) and 2.7 kb (Tus-blocked) fragments at the origin (Fig. 5C, before release from *lacO*₂₂). This ligation competes with SmaI cleavage at the origin making it incomplete in our termination assay; however, we

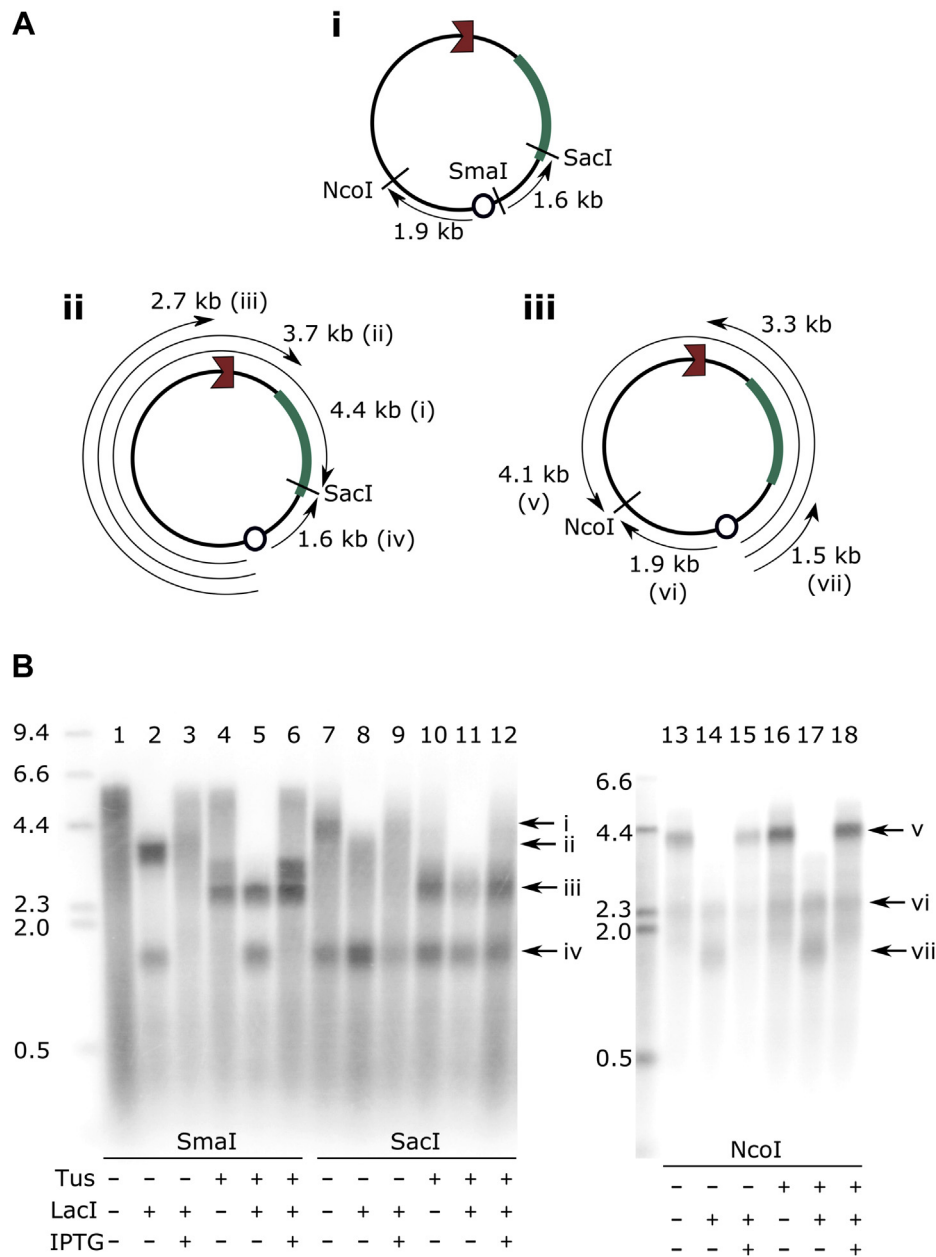


Figure 4. The unreplicated gap at *terB* is a direct consequence of replisomes meeting at Tus-ter. Analysis of unidirectional replisome travel toward Tus-*terB* (A) (i) Locations of SmaI, NcoI, and SacI, with respect to the replication origin. ii, hypothetical leading strand products if the template is linearized by SacI cleavage. iii, hypothetical leading strand products if the template is linearized by NcoI cleavage. B, denaturing agarose gel of replication products linearized at SmaI, SacI, or NcoI as indicated. Band labels i to vii refer to replication products shown in (A). Replication products from templates linearized by SmaI are fully described in Figure 2, B–E and reproduced here (lanes 1–6) for comparison with SacI and NcoI linearizations. Lanes 7 to 18 are representative of three replicates. Replication reactions contain 40 mM HEPES pH 8.0, 10 mM Magnesium Acetate, 10 mM DTT, 2 mM ATP, 200 μ M GTP/UTP/CTP, 40 μ M dNTPs, 0.1 mg/ml BSA, 200 ng template DNA (approx. 2 nM), 50 nM polymerase III core ($\alpha\epsilon\theta$), 25 nM clamp loader complex ($\tau_3\delta\delta'\chi\psi$), 160 nM DnaB, 160 nM DnaC, 1 μ M SSB, 80 nM β -clamp, 30 nM HU, and 200 nM DnaG, in a final volume of 15 μ l. Where indicated, 400 nM LacI, 400 nM Tus, or both proteins are present at the start of the reaction. Reactions are initiated by addition of 300 nM DnaA but can only progress approx. 1 kb in either direction (no gyrase present). After 2 min of active replication, 30 units of SmaI (Promega) (lanes 1–6) or 30 units SacI-HF (NEB) (lanes 7–12) or 30 units NcoI-HF (NEB) (lanes 13–18) were added to release the active fork, alongside 46 kBq [a - 32 P]-dCTP (111 TBq/mmol). Where indicated, 1 mM IPTG is also added after 2 min active replication. Reactions are stopped after 4 min active DNA replication.

also carried out control reactions in the presence of RLP, Tus and LacI in the absence of SmaI, to assess the ability of RLP to ligate adjacent nascent DNA products. In this instance, RLP is able to promote near complete ligation of the 1.5 kb and 2.7 kb products into a 4.2 kb product at the same concentrations used in our controlled termination assay (Fig. S2). This further

indicates that we would expect to see a much more substantial increase in full-length products if RLP were able to promote proper termination and ligation of converging products formed at Tus-*ter*. Overall, these data indicate that the lagging strand processing enzymes are insufficient to resolve termination events at Tus-*ter*.

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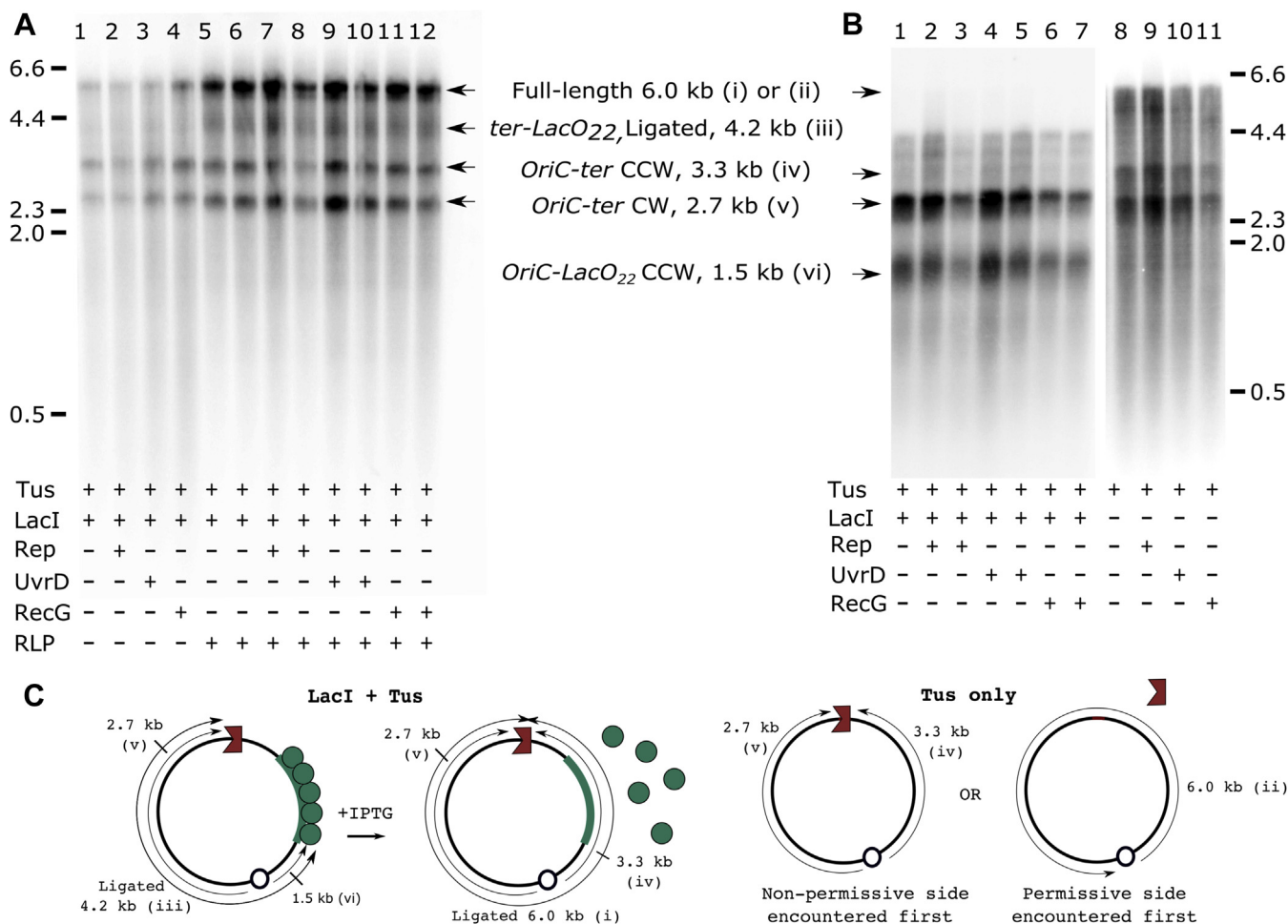


Figure 5. Rep, UvrD, or RecG does not assist replication fork fusion at Tus-ter *in vitro*. A, denaturing agarose gel of replication products from controlled fusion reactions at Tus-ter. Replication reactions contain 40 mM HEPES pH 8.0, 10 mM Magnesium Acetate, 10 mM DTT, 2 mM ATP, 200 μ M GTP/UTP/CTP, 40 μ M dNTPs, 0.1 mg/ml BSA, 200 ng template DNA (approx. 2 nM), 50 nM polymerase III core ($\alpha\epsilon\theta$), 25 nM clamp loader complex ($\tau_3\delta\delta'\chi\psi$), 160 nM DnaB, 160 nM DnaC, 1 μ M SSB, 80 nM β -clamp, 30 nM HU and 200 nM DnaG, 133 nM Gyrase (A_2B_2), and 400 nM LacI and 400 nM Tus in a final volume of 15 μ l. Where indicated, 4 nM RNase HI, 25 nM DNA Ligase, and 30 nM DNA Polymerase I (RLP) and/or 200 nM Rep, UvrD, or RecG are included from the start. Reactions are initiated by addition of 300 nM DnaA. After 2 min of active replication, 30 units of SmaI (Promega), 46 kBq [a - 32 P]-dCTP (111 TBq/mmol), and 1 mM IPTG are added to the reactions. Reactions are stopped after 4 min active DNA replication. Addition of RLP in the presence or absence of Rep, UvrD, or RecG is unable to increase the proportion of full-length replication products. B, denaturing agarose gel of replication products blocked at Tus-ter and LacI-*lacO₂₂*. Addition of Rep, UvrD, or RecG does not assist replisome progression through Tus-ter. Reactions are carried out as described in (A). Only where indicated, reactions include 400 nM Tus and/or 400 nM LacI from the start. Also where indicated, reactions include 200 nM Rep, UvrD, or RecG from the start. IPTG is omitted in all reactions. C, predicted replication products from reactions containing Tus and LacI or Tus-only. Replication product labels correspond to labeled bands in panels A and B.

The replication gap cannot be closed by the accessory helicases Rep and UvrD or by the helicase RecG

Several *E. coli* helicases have been associated with successful DNA replication events *in vivo*. The accessory helicases Rep and UvrD are known to assist replisome progression by removing barriers which would otherwise impede oncoming replisomes (35, 36). Of note, UvrD has previously been implicated in overcoming Tus-ter barriers both *in vivo* (28) and *in vitro* (27). In addition, the helicase RecG appears to play a role in the successful termination of DNA replication *in vivo* (17, 25). We therefore hypothesized that the addition of one of these helicases to our assay may facilitate processing of the gap in replication we observed at Tus-ter. We first investigated whether Rep, UvrD, or RecG could overcome the Tus-ter barrier when the replisome approaches Tus-ter in a nonpermissive direction. We saw no progression beyond Tus-ter

when replisomes were blocked in both directions (Fig. 5B, lanes 2–7 and Fig. 5C) and no increase in full-length products compared with a control in the absence of the helicases when replisomes are blocked only by Tus (Fig. 5B, compare lane 8 with lanes 9–11, and Fig. 5C). This indicates that the helicases were unable to assist the replisome in progression through the Tus-ter barrier.

We next added Rep, UvrD, or RecG to our fusion assay where one replisome is released at *lacO₂₂* to meet the other replisome at Tus-ter. Although the helicases are unable to overcome the Tus-barrier in the nonpermissive direction, we reasoned they may be able to remove Tus in the permissive direction in our full replication assay, facilitating strand fusion. Again, we analyzed the proportion of full-length replication products compared with all replication products in the presence of RLP. Addition of UvrD, Rep, or RecG only produced a

minor increase in observed full-length products (Fig. 5A, compare lanes 5 and 6 with 7–12) and no increase beyond that observed for the addition of RLP alone (Table S1). Densitometric analysis of the gel lanes confirmed that a greater proportion of the lane density was incorporated into the bands containing RLP due to ligation of lagging strands contributing to each product (Fig. S3). To account for this, we repeated our analysis of band intensities; comparing each band to the total intensity attributable to all bands instead of that of the entire lane, making it easier to compare the proportion of products contributing to each band (Table S2). Again, this analysis indicated only a slight shift in the proportion of full-length product (+10%) in the presence of RLP (Fig. S3B and Table S2). Again, we suspect this may be a consequence of off-target ligase activity in the assay. Overall, these results demonstrate that neither UvrD, nor Rep, nor RecG was able to facilitate strand fusion at Tus-ter, leaving open the question of how the observed gap is closed in living *E. coli* cells.

Discussion

Termination of DNA replication in bacteria occurs when two replisomes translocating away from the origin in opposite directions meet in the termination area opposite *oriC*. For a successful termination event to occur, nascent leading and lagging strands need to be ligated, the replisomes disassembled, and the resulting fully replicated chromosomes decatenated by topoisomerases. In *E. coli*, the majority of fusion events occur at the chromosomal midpoint away from Tus-ter sites, indicating that both replisomes travel with approximately the same speed (16, 17). However, replisomes can get delayed at obstacles such as a nucleoprotein block, DNA secondary structures, or DNA lesions. If one fork is significantly delayed, then the second fork will proceed until it is paused at a nonpermissive Tus-ter face. This fork will remain stalled at Tus-ter until the delayed replisome arrives on the permissive side, thereby forcing termination to occur directly at a Tus-ter complex. Forks blocked at Tus-ter complexes can be easily visualized in normally growing cells, highlighting that they are a regular occurrence (26).

Our characterization of a fusion event at a Tus-terB complex *in vitro* has highlighted an inability for established replisome components to complete replication termination at Tus-terB on their own, leaving a gap of at least 15 bp of unreplicated DNA across the *terB* site. Given the similarity of the *E. coli* *ter* sites and particularly those most commonly used *in vivo*, the innermost sites *terC*, *terA*, and *terB* (26), we believe that our *in vitro* results accurately represent the physiological situation for termination events at Tus-ter *in vivo*.

In an analogous plasmid-based replication system, it has previously been reported that the inclusion of a Tus-ter replication fork trap (where both replisomes approach Tus-ter in the permissive direction) prevents the formation of fully replicated circular monomeric products when the fork trap is included close to the predicted fusion point (27). In this report, fully replicated products could only be produced in conditions where both replisomes were likely to have already translocated

beyond Tus-ter at the point of convergence (27). This implies that fusion at Tus-ter was also responsible for preventing complete replication in this system. The mapping analysis we have carried out here strongly indicates that the failure to produce complete replication products in the presence of a replication fork trap is a direct result of a gap in nascent replication products when replisomes meet at Tus-ter. Thus our results indicate that additional protein activities are necessary to complete DNA replication if one fork is paused at a Tus-ter complex and the second fork approaches from the permissive side. Indeed, the analysis of genetic data has led to the hypothesis that a fusion event between two freely moving forks will generate very different intermediates to the situation where one replisome blocked at the nonpermissive face of Tus-ter fuses with a freely moving replication fork complex that approaches from the opposite direction (23, 26) However, we previously observed that when DNA replication is forced to terminate at Tus-ter due to an additional ectopic replication origin, the majority of cells grow without much ill-effect (37, 38). Thus, cells are able to process this type of fusion event without much difficulty.

Two obvious additional requirements for the fusion of replication forks at a Tus-ter complex are the displacement of Tus from the *ter* site and the replication of the *ter* site itself prior to the ligation of leading and lagging strands. The enzymes DNA Polymerase I, RNase HI, and DNA Ligase are responsible for replacing RNA primers with DNA and joining adjacent lagging strands to one another during DNA replication. As a directly equivalent process, they would also be anticipated to ligate the nascent leading strand with the final lagging strand produced by converging replisomes during the termination of DNA replication. Yet, the addition of DNA Polymerase I, DNA Ligase, and RNase HI did not result in the gap being closed in our controlled fusion reactions, suggesting that at least one additional processing factor is necessary to allow DNA replication to successfully complete at Tus-ter.

It has been established that Tus is removed from *ter* when approached by a replisome (or the replicative helicase) from the permissive direction (12, 13). However, to our knowledge, whether the same applies when another replisome is already blocked at the nonpermissive face has not been determined. We observed a marked similarity between the Tus-binding footprint and the unreplicated gap observed in our controlled fusion reactions (Fig. 3D). At first glance, the final two leading strand stop sites on the permissive side appear to overlap with the Tus-binding footprint, but closer analysis of the Tus-ter locked structure (12) shows that the leading strand template at these stop sites makes very few contacts with Tus and the majority of these bases are solvent-exposed. In fact, it is the lagging strand template that is much more tightly bound by Tus on the permissive side. This suggests that it is possible for the leading strand template to be available up to the final stop residue without Tus being removed from the DNA. This possibility is especially easy to envisage if the *E. coli* replisome is topologically similar to that of the T7 replisome, where both helicase and polymerase have recently been shown to sit at the head of the replisome, synergistically unwinding the DNA

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duplex (39). We envisage two possible scenarios as the replisome approaches Tus on the permissive side. In the first, the converging replisome has not yet reached Tus on the nonpermissive side and the replisome approaching Tus at the permissive side is able to displace Tus by pushing forward, causing a loss of DNA specific interactions and dissociation from the DNA. However, in the second, the converging replisome has already been stably arrested at Tus on the nonpermissive side; making displacement impossible and meaning that Tus becomes sandwiched between the two replisomes.

If the replisome is unable to remove Tus itself, what additional factor is required to displace Tus and allow replication to be completed? It has been previously noted that *E. coli* sometimes appears to be able to overcome Tus-ter barriers *in vivo* (40). Likely candidates for this role are the *E. coli* accessory helicases Rep and UvrD. Numerous investigations have been carried out previously to understand if Rep or UvrD is able to displace Tus when traveling toward the nonpermissive interface, with contradictory results (41–44). In some experimental conditions, both proteins appear to be blocked by Tus (41) or able to displace Tus (44) when approaching the Tus nonpermissive interface, while others showed that Rep (42) or UvrD (43) was able to displace Tus. UvrD has been further implicated as a candidate for displacing Tus *in vivo* by a report that the gene is required for viability of a strain carrying ectopic *ter* sites designed to block normal replisome progression (28). UvrD was also able to promote the formation of circular replication products in a plasmid-based replication system containing a replication fork trap, where complete replication was otherwise prevented by the addition of Tus (27). However, when we included these proteins in our reconstituted replisome fusion assays, we did not observe any ability of Rep or UvrD to displace Tus when approaching in the nonpermissive direction. This is a striking difference to the ability of Rep and UvrD to displace RNA polymerase and facilitate replisome progression in a directly analogous assay (35). Moreover, Rep and UvrD were unable to promote successful ligation of leading and lagging strands from replisomes converging at Tus-ter. These results strongly suggest that they are either not responsible for displacing Tus *in vivo* or that an additional activating factor and/or helicase cooperativity is necessary for them to be able to do so. Notable differences in the reactions performed in (27) and the assays here are the addition of the helicase RecQ and topoisomerases III and IV. Template linearization in our assay should prevent any topological stress and remove the need for topoisomerases, which suggests that the helicase activity of RecQ is the main difference between these assays. An inability to complete DNA replication is common to both studies, and our data indicate that the problem may be caused by the replisomes–Tus supercomplex. Our system will be valuable for identifying helicase cooperativity and/or helicase activating factors required to overcome Tus-ter barriers during fork fusion.

Another helicase implicated in facilitating fork fusion is RecG. RecG has been shown to play a role in processing intermediates, which result from fusion events that otherwise

result in replication restart from the terminus region. However, RecG was neither able to displace Tus from the DNA nor promote ligation of strands during fusion at Tus-ter *in vitro*. This again suggests that another unknown molecule, or stimulatory factor, is required for successful termination at Tus-ter. Indeed, in cells that carry an additional ectopic replication origin, which results in one replication fork complex almost always being blocked at Tus-ter, it has been shown that both RecG and Rep can be inactivated without much ill-effect to the cells (25, 35).

Our results pose the obvious question as to the evolutionary advantage of Tus-ter for the *E. coli* cell. A recent analysis has shown that sequences related to Tus are found in most Enterobacteriales, in the Pseudoalteromonas, and in most Aeromonadales (20). In most other bacterial species, there is no replication fork trap present, as experimentally demonstrated for the two circular *Vibrio cholerae* chromosomes (20). Thus, it appears that the majority of bacterial species have little difficulty surviving without a replication fork trap. Indeed, a fork trap introduces a significant level of constraint to chromosome duplication: if one fork is stalled at an obstacle before it has progressed through the first five *ter* sites it encounters and cannot be reactivated, the second fork will be unable to rescue this blocked fork because it will be blocked by Tus-ter complexes. If the stalled fork cannot be reactivated, the cells are in danger of dying (3, 8), a problem that will not arise in the same way in bacterial chromosomes without a fork trap. We were recently able to recreate this scenario *in vivo*, by moving the origin from its original location into either the right-hand or left-hand replichore (37, 38). In these cells, replisomes coming from ectopic origins have to duplicate a significant proportion of the chromosome in an orientation opposite to normal, which results in replication–transcription conflicts that will delay the progression of one fork, while the second fork is stalled at Tus-ter complexes (8, 37, 38). The resulting cells show a significant growth defect, indicating that this scenario can cause serious issues for the cell. The observed growth defect is significantly alleviated if Tus is absent, demonstrating that preventing forks from being trapped is advantageous (8, 37, 38).

The results of this study highlight yet another constraint that is introduced by the replication fork trap: processing beyond that provided by standard replisome components is required when two replisomes converge at Tus-ter, otherwise the chromosome will remain under-replicated (Fig. 3). While the majority of fork fusions take place away from Tus-ter complexes *in vivo* (16, 17), delays by one of the two replisomes are frequent enough to allow the detection of forks stalled at Tus-ter complexes (26). Thus, cells deal with this scenario on a regular basis. The fork trap must therefore provide a significant advantage to cells in order to compensate for the difficulties it can cause. The nature of this advantage is still unclear. Our previous results suggest that toxic intermediates arise in the termination area due to fork-fusion events. These intermediates are normally efficiently processed by proteins such as RecG helicase and 3' exonuclease, but in their absence, lead to significant over-

replication, which is contained by the replication fork trap (3, 17). These intermediates can trigger cell lethality, leading to the speculation that the main purpose of the replication fork trap is to ensure that they only arise and are contained to a defined area of the chromosome where they can be efficiently processed (3). Cells without a termination area still have all the necessary proteins to process intermediates that might arise as a result of fork fusion events. However, acquiring the fork trap system has provided cells with the advantage of being able to confine the intermediates to a defined area of the chromosome, an advantage apparently strong enough to compensate for the constraints it introduces.

In conclusion, we have demonstrated that the fusion of two replication forks at Tus-ter results in incomplete DNA replication, leaving a gap of at least 15 bp of unreplicated DNA. Additional processing beyond that provided by established replisome components must be required for successful termination. The most obvious candidates, Rep, UvrD, and RecG, are unable to promote replication to completion in this scenario, suggesting that another as yet unidentified molecule is likely to participate in replication termination at Tus-ter. Further investigation is required to understand the comprehensive requirements for completing DNA replication at Tus-ter, for understanding how and when Tus is displaced from the DNA during fusion and how the final stretch of DNA across the *ter* site is replicated. Such investigations will not only shed light on the molecular mechanics of the termination process, but also the precise reason why acquiring a replication fork trap is advantageous. Ultimately, they will lead to a better understanding of the factors that have shaped the overall landscape of bacterial chromosomes.

Experimental procedures

Plasmids

Backbone fragments of pKJ1 were purchased from Thermo Fisher Genestrings service. Two dsDNA fragments of 2269 and 2945 bp were synthesized based on pIK02 (32). The fragments were designed to retain the *E. coli* replication origin, *oriC*, the plasmid initiation site *colE1*, the Tus DNA-binding site, *terB*, and the ampicillin resistance gene, *bla*. Recognition sequences for four unique ssDNA nicking enzymes were included, two approximately 100 bp upstream and two approximately 100 bp downstream of *terB* (Fig. 3A). All other instances of these ssDNA nicking sequences were removed in the design of the synthetic fragments. A repeat region of DNA containing 22 *lacO* binding sites was amplified from pIK02 by PCR using the following primers: 5'-GCCAGCACGTAGCTAGCAAACCG-3' and 5'-CCTTCTAGAGATTCCGACTCTAGAGTCC-3'. Fragments were annealed by ligation-independent cloning using T4 polymerase (NEB) to generate complementary 5'-overhangs, as described (45).

Protein production

Proteins used in replication assays were purified as previously described (46). Rep and UvrD were purified as

described (47). RecG was a kind gift from Robert Lloyd and Geoff Briggs. RNase HI, DNA Ligase, and DNA Polymerase I encoding genes (*rnhA*, *ligA* and *polA* respectively) were cloned into a modified pET28a vector, which encodes an N-terminal His₆SUMO-tag. Proteins were overexpressed in BL21 (DE3). Cells were grown at 37 °C to an OD₆₀₀ of 0.6 to 0.8 before expression was induced by the addition of 1 mM IPTG. Growth was continued at 18 °C for approximately 18 h before cells were harvested by centrifugation and resuspended in a buffer of 50 mM Tris pH 8.0, 500 mM NaCl, 1 mM DTT, and 20 mM imidazole. Cells were lysed by sonication and cell debris pelleted by centrifugation. DNA was precipitated from soluble cell lysate by the dropwise addition of Polyethylenimine to a final concentration of 0.075% (v/v) and stirring for 20 min at 4 °C. Precipitated DNA was pelleted by centrifugation and soluble lysate was loaded onto a 5 ml HisTrap HP column (GE Healthcare). The column was washed with seven column volumes of loading buffer before being eluted with a gradient of imidazole (20–500 mM) over 20 column volumes (100 ml). Fractions corresponding to the presence of the protein of interest were combined according to chromatographic analysis at A280 and SDS-PAGE analysis. The His₆SUMO tag was cleaved by the addition of ULP1 and incubation overnight at 4 °C. The protein was simultaneously dialyzed into 50 mM Tris pH 8.0, 500 mM NaCl, 1 mM DTT to remove imidazole from the buffer. Cleavage products were passed over a second HisTrap HP column, which was washed and eluted in an analogous manner to the first. Flow-through fractions containing the protein of interest were combined and concentrated to <1 ml using a centrifugal concentrator. Proteins were further purified by gel filtration. RNase HI was loaded onto a 16/600 Superdex 75 column (GE Healthcare), while PolI and Ligase were further purified on a 16/600 Superdex 200 column (GE healthcare). Following gel filtration, Ligase was additionally dialyzed into 50 mM Tris pH 7.5, 75 mM NaCl, 1 mM DTT and loaded onto a 1 ml HiTrap Q column and eluted with a gradient of salt (75–1000 mM NaCl) over 30 column volumes. RNase HI, DNA Ligase, and PolI were dialyzed into a storage buffer of 50 mM Tris pH 7.5, 100 mM NaCl, 2 mM DTT, 40% glycerol before storage at –80 °C. Protein concentrations were calculated by measuring absorbance at 280 nm using a Nanodrop photospectrometer (Thermo Scientific) and modified according to the Beer–Lambert law. Protein extinction coefficients at 280 nM were calculated using the ExPASy ProtParam web server, assuming all cysteine residues are reduced (48). The extinction coefficient for DNA Ligase was 35,410 M⁻¹ cm⁻¹; for RNase HI, 40,450 M⁻¹ cm⁻¹; and for PolI, 86,180 M⁻¹ cm⁻¹. Tests were carried out to confirm protein activities in replication assay buffer conditions. No protein showed significant dsDNA nuclease activity over the timeframe of the assays used in this study. Only SSB exhibited unexpected ssDNA 3' exonuclease activity (Fig. S1) over the timescale of the assays used in this study. No free 3' single-stranded ends exist during DNA replication because the nascent strand is protected by its location within the polymerase active site and becomes

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double stranded after base addition. Therefore this contaminating exonuclease activity does not affect any results.

Replication assay

Replication reactions were carried out in 40 mM HEPES pH 8.0, 10 mM Magnesium Acetate, 10 mM DTT, 2 mM ATP, 200 μ M GTP/UTP/CTP, 40 μ M dNTPs, and 0.1 mg/ml BSA. Each reaction (15 μ l final volume) contained 200 ng template DNA (approx. 2 nM), 50 nM polymerase III core ($\alpha\epsilon\theta$), 25 nM clamp loader complex ($\tau_3\delta\delta'\chi\psi$), 160 nM DnaB, 160 nM DnaC, 1 μ M SSB, 80 nM β -clamp, 30 nM HU, 200 nM DnaG, and 133 nM Gyrase (A_2B_2). Where indicated, reaction mixes contain 400 nM Tus and/or 400 nM LacI. Reaction mixes were assembled on ice, then incubated at 37 °C for 2 min before replication was initiated by addition of 300 nM DnaA. Reactions were then incubated at 37 °C for 2 min before the addition of 30 units of SmaI (Promega), 46 kBq [a - 32 P]-dCTP (111 TBq/mmol), and where indicated, 1 mM IPTG. Reactions were incubated for a further 2 min at 37 °C before being terminated by the addition of 2.5 μ l STOP buffer (2.5% SDS, 200 mM EDTA, and 10 mg/ml Proteinase K). Reaction products were precipitated by ethanol precipitation and resuspended in 25 μ l 50 mM NaOH, 30 mM EDTA before analysis by denaturing gel electrophoresis (0.7% agarose in 30 mM NaOH, 2 mM EDTA). 5'-labeled HindIII-digested λ DNA was used as a marker. Samples were run on a 40 cm gel for 400 Vh (typically 16 h at 25 V) before being dried and analyzed by phosphorimaging and autoradiography. One-way assays were carried out as above except for the exclusion of Gyrase and SmaI was substituted for 30 units NcoI-HF (NEB) or 30 units SacI-HF (NEB). Reactions containing RLP include 4 nM RNase HI, 25 or 50 nM DNA Ligase and 30 nM DNA Polymerase I. These concentrations were based on studies from the Marians group (29) and enzyme activity tests (Fig. S2). Where indicated, Rep, UvrD, or RecG was included at a concentration of 200 nM. Concentrations of these helicases were chosen based on active concentrations in similar replication assays (35, 49, 50).

Termination mapping

Replication reactions to map replication end points were carried out as described above with a 4 \times reaction mix (60 μ l). Following ethanol precipitation, reaction products were resuspended in 50 μ l relevant manufacturer's enzyme buffer (1 \times Cutsmart (NEB) for nicking with Nt.BspQI or 1 \times Buffer R (Thermo Fisher) for nicking with Nb.Bpu101). Products were subsequently split into two 25 μ l aliquots; one of which was incubated with 1 μ l nicking enzyme (10 units Nt.BspQI (NEB) or 5 units Nb.Bpu101 (Thermo Fisher)) and the other, 1 μ l enzyme buffer (control). Reactions were incubated at 37 °C for 30 min, then at 80 °C for 20 min. Products were split into two 12.5 μ l samples. To one sample, 6 μ l STOP solution (USB Thermo Sequenase Cycle Sequencing Kit, Affymetrix) was added for analysis by Urea PAGE gel electrophoresis. The other sample was precipitated by ethanol precipitation and resuspended in 12.5 μ l 50 mM NaOH, 30 mM EDTA for

analysis by denaturing gel electrophoresis (see Replication assay above).

Cycle sequencing reactions were carried out according to the manufacturer's instructions (USB Thermo Sequenase Cycle Sequencing Kit, Affymetrix) with minor modifications. In brief, primers were 3'-labeled with [a - 32 P]-dCTP in reaction mixes containing dGTP dATP but excluding dTTP. 3'-labeling was carried out using thermocycling between 95 °C (15 s) and 60 °C (30 s) for 50 cycles. The termination step was also carried out in the presence of all four dNTPs and dd-NTPs for chain termination. During the termination step, reactions were thermocycled between 95 °C (30 s) and 72 °C (90 s) for 50 cycles. The leading strand approaching the Tus-terB nonpermissive face (clockwise travelling replisome) was sequenced with the primer 5'-AATGCTTATTATCATGACATTACCTATCC-3' while the leading strand approaching Tus-terB permissive face (counterclockwise traveling replisome) was sequenced with the primer 5'-TAAGGCATTTGTTTCAGGTTACTCC-3'.

Nicked reaction products and sequencing products were analyzed alongside one another by 8% Urea TBE PAGE. Gels were run in 1% TBE at 50 W for 120 min. Gels were dried and analyzed by phosphorimaging and autoradiography.

Data availability

Data not included in the manuscript or supporting information can be obtained on request by contacting the corresponding author (Michelle Hawkins: michelle.hawkins@york.ac.uk).

Supporting information—This article contains supporting information.

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