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Interplay between chromosomal architecture and termination of DNA replication in bacteria

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Faithful transmission of the genome from one generation to the next is key to life in all cellular organisms. In the majority of bacteria, the genome is comprised of a single circular chromosome that is normally replicated from a single origin, though additional genetic information may be encoded within much smaller extrachromosomal elements called plasmids. By contrast, the genome of a eukaryote is distributed across multiple linear chromosomes, each of which is replicated from multiple origins. The genomes of archaeal species are circular, but are predominantly replicated from multiple origins. In all three cases, replication is bidirectional and terminates when converging replication fork complexes merge and 'fuse' as replication of the chromosomal DNA is completed. While the mechanics of replication initiation are quite well understood, exactly what happens during termination is far from clear, although studies in bacterial and eukaryotic models over recent years have started to provide some insight. Bacterial models with a circular chromosome and a single bidirectional origin offer the distinct advantage that there is normally just one fusion event between two replication fork complexes as synthesis terminates. Moreover, whereas termination of replication appears to happen in many bacteria wherever forks happen to meet, termination in some bacterial species, including the well-studied bacteria *Escherichia coli* and *Bacillus subtilis*, is more restrictive and confined to a 'replication fork trap' region, making termination even more tractable. This region is defined by multiple genomic terminator (*ter*) sites, which, if bound by specific terminator proteins, form unidirectional fork barriers. In this review we discuss a range of experimental results highlighting how the fork fusion process can trigger significant pathologies that interfere with the successful conclusion of DNA replication, how these pathologies might be resolved in bacteria without a fork trap system and how the acquisition of a fork trap might have provided an alternative and cleaner solution, thus explaining why in bacterial species that have acquired a fork trap system, this system is remarkably well maintained. Finally, we consider how eukaryotic cells can cope with a much-increased number of termination events.

KEYWORDS

termination of DNA replication, DNA replication, DNA segregation, RecG helicase, Tus-*ter* complexes, bacterial chromosome dynamics, chromosomal architecture

Introduction

Bacteria show a considerable degree of variability in terms of genome size and organization. Genomes can be as small as 0.159 Mbp, such as in the symbiotic bacterium *Carsonella ruddii*, which carries only 182 genes (Nakabachi et al., 2006), and as large as 13 Mbp in the myxobacterium *Sorangium cellulosum*, with 9,376 predicted open reading frames (Schneiker et al., 2007). The majority of bacteria carry a single chromosome, in the form of a covalently closed circle. About 5% carry multiple chromosomes (Touchon and Rocha, 2016). These species include *Vibrio cholerae* and close relatives, with two (Touchon and Rocha, 2016) and *Paracoccus denitrificans*, with three (Winterstein and Ludwig, 1998). A few species in the *Actinomycetales*, including the genus *Streptomyces* (Kirby, 2011), have linear chromosomes or a mixed state. For instance, *Agrobacterium tumefaciens* has one circular and one linear chromosome, along with two mega-plasmids (Nester, 2015).

In all bacteria, replication initiation is tightly regulated (Jameson and Wilkinson, 2017; Katayama et al., 2017; Hansen and Atlung, 2018) and restricted to a single origin (*oriC*) per chromosome (Gao and Zhang, 2008; Gao, 2015). Two replisomes are recruited at *oriC* by the main initiator protein, DnaA, forming two replication fork complexes that bidirectionally duplicate the genome. Bidirectional synthesis continues until the replication fork complexes fuse in an area opposite *oriC* where replication terminates (Masters and Broda, 1971; Prescott and Kuempel, 1972; Wake, 1972, 1973; Gyurasits and Wake, 1973). For bacteria with linear chromosomes replication ceases when fork complexes reach the chromosome ends (Cui et al., 2007; Rudolph et al., 2013). In both cases, the chromosome is divided into two replichores, each replicated with a defined directionality (Figure 1A).

The fact that replication is restricted to two replication fork complexes dictates that, for bacteria with a circular chromosome, each round of chromosome duplication is concluded by exactly one termination event in an area opposite *oriC*. For termination two main mechanisms were initially considered in the literature: the free fusion of moving forks, or site-specific termination. Forks may proceed around the chromosome freely and fuse wherever they happen to meet. Alternatively, there might be distinct locations where fork progression is blocked, forcing fork fusion to take place in a distinct location (Masters and Broda, 1971; Bird et al., 1972; Hendrickson and Lawrence, 2007; Kono et al., 2012).

Replication fork complexes will encounter a wide variety of potential blocks on their way to the terminus area, such as stable protein-DNA complexes, G4 quadruplex DNA or DNA lesions (Lang and Merrih, 2018; Marians, 2018; Bianco, 2020; Linke et al., 2021). Many of these obstacles are stochastic in nature and can occur at different chromosomal locations for each round of genome duplication. However, there are also mechanisms that have specifically evolved to arrest replication complexes (Hyrien, 2000; Rothstein et al., 2000; Labib and Hodgson, 2007; Hizume and Araki, 2019). The first examples of distinct blocking points for replication complexes were identified in plasmid systems. Replication of the conjugative R plasmid R6K was reported to be either unidirectional or bidirectional (de Graaff et al., 1978), based on a complex mechanism of replication initiation (Abhyankar et al., 2003, 2004; Rakowski and Filutowicz, 2013), but progression of both the clockwise and counter-clockwise forks is blocked by a specific terminus (Crosa et al., 1976). In the R1 plasmid, a distinct termination system is in use to specifically enforce

unidirectional replication. Between the minimal origin, *oriR*, and the start site for leading strand replication, two *ter* sites are located, *terR_L* and *terR_R* (Hill et al., 1988b). *terR_L* blocks the counter-clockwise fork almost immediately. Thus, the plasmid is replicated unidirectionally via the clockwise fork until it is blocked at *terR_R* (Krabbe et al., 1997).

The first chromosomal fork block was identified in *E. coli* (Kuempel et al., 1977; Louarn et al., 1977), and shortly afterwards in *B. subtilis* (Weiss et al., 1981). In *E. coli* the integration of an ectopic replication origin into the chromosome was used to show that progression of replication forks was blocked in the termination area (Kuempel et al., 1977; Louarn et al., 1977, 1979). By using a temperature-sensitive version of the main replication initiator protein DnaA to inactivate *oriC*, and inserting an ectopic P2 prophage origin to drive replication initiation at a site 1 Mbp away from *oriC*, the authors showed that termination still occurred diametrically opposite *oriC*, revealing that progression of replication must be impeded in an area opposite *oriC* (Kuempel et al., 1977; Louarn et al., 1977, 1979). Prompted by these results, the use of sophisticated marker frequency analysis revealed specific termination sites, initially called T1 and T2 and later changed to *ter*, in line with plasmid-based systems such as R6K and R1, with the Louarn group identifying the innermost *ter* sites *terA* and *terC*, and the Kuempel lab identifying *terA* and *terB* (Figure 1A; de Massy et al., 1987; Hill et al., 1987, 1988a; Hill and Marians, 1990). In addition, the Kuempel lab identified a region close to *terB* that was needed for *ter* sites to act as a block, and they named the terminus function *termination utilization substance* (*tus*) (Hill et al., 1988a). Further analysis revealed the sequence of the *tus* gene (Hill et al., 1989), and *in vitro* work by Thomas Hill and Ken Marians showed how the binding of Tus protein to a *ter* sequence can arrest progression of DNA replication (Hill and Marians, 1990; Figure 1B).

By developing *ter* consensus sequences, the use of radioactively labeled probes allowed identification of additional *ter* sites *D*, *E*, and *F* (François et al., 1989; Hidaka et al., 1991; Sharma and Hill, 1992). Once the whole genome sequence of *E. coli* was available (Blattner et al., 1997), bioinformatics analysis resulted in the identification of *terG-terJ* (Neidhardt et al., 1996). Thus, the *E. coli* chromosome is divided into two replichores, one replicated clockwise and the other counter-clockwise, with 5 *ter* sites being oriented to block progression of the clockwise replication fork, while the other 5 *ter* sites block the counter-clockwise moving replication fork complex (Figure 1A). Finally, bioinformatics analysis also allowed the identification of the putative and weak *ter* sites *K*, *L*, *Y*, and *Z* (Duggin and Bell, 2009).

The specific termination site identified in the Gram-positive *Bacillus subtilis* (Weiss et al., 1981) was later characterized to consist of a cluster of *ter* sites blocking progression of the clockwise replication fork complex, while a second cluster blocked progression of the counter-clockwise replication fork complex (Wake, 1997; Neylon et al., 2005; Rudolph et al., 2019). While acting in a similar fashion, the *ter* sequences show very little similarity to the *E. coli* sequences. Moreover, the *B. subtilis* terminator protein, RTP, has little structural similarity to Tus. Importantly, these differences indicate that the two termination systems developed *via* convergent evolution (Franks et al., 1995; Neylon et al., 2005).

In this review we will discuss how the architecture of bacterial chromosomes limits pathological consequences that can arise when replication forks fuse and explore why a fork trap in the termination area might provide an evolutionary benefit by limiting the local and global impacts caused by fork fusions. Based on the pathological

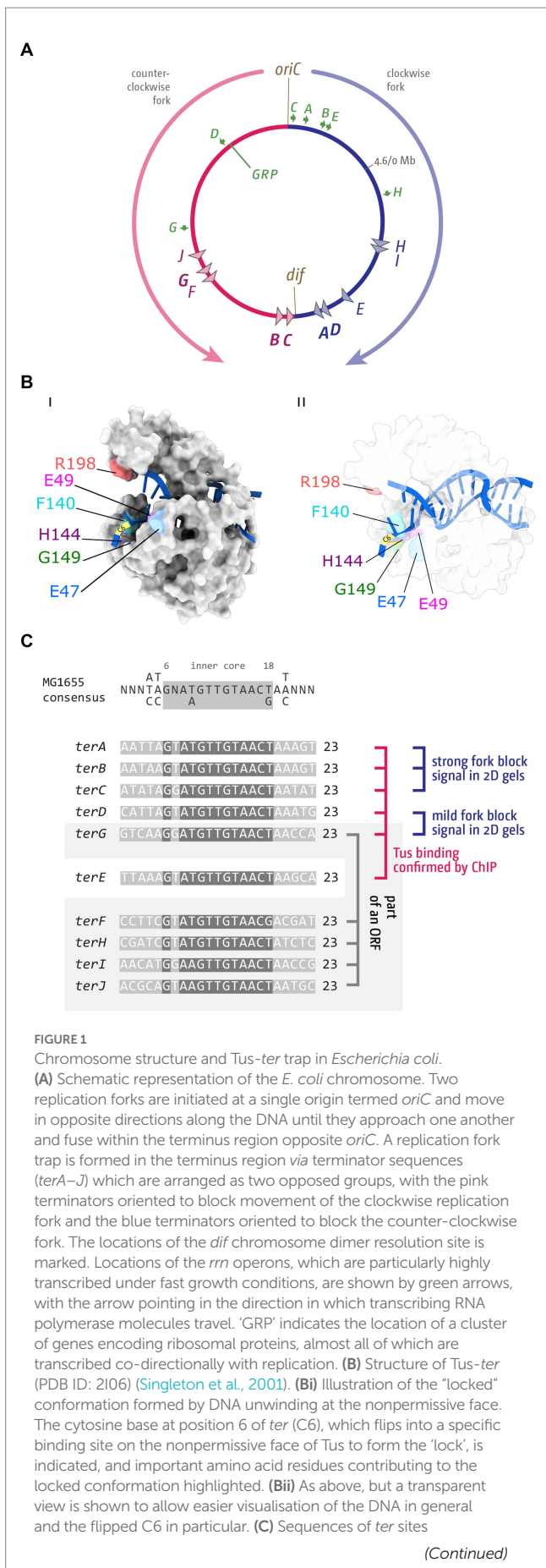


FIGURE 1
Chromosome structure and Tus-ter in *Escherichia coli*. (A) Schematic representation of the *E. coli* chromosome. Two replication forks are initiated at a single origin termed *oriC* and move in opposite directions along the DNA until they approach one another and fuse within the terminus region opposite *oriC*. A replication fork trap is formed in the terminus region via terminator sequences (*terA–J*) which are arranged as two opposed groups, with the pink terminators oriented to block movement of the clockwise replication fork and the blue terminators oriented to block the counter-clockwise fork. The locations of the *dif* chromosome dimer resolution site is marked. Locations of the *rrn* operons, which are particularly highly transcribed under fast growth conditions, are shown by green arrows, with the arrow pointing in the direction in which transcribing RNA polymerase molecules travel. ‘GRP’ indicates the location of a cluster of genes encoding ribosomal proteins, almost all of which are transcribed co-directionally with replication. (B) Structure of Tus-ter (PDB ID: 2I06) (Singleton et al., 2001). (Bi) Illustration of the “locked” conformation formed by DNA unwinding at the nonpermissive face. 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, and important amino acid residues contributing to the locked conformation highlighted. (Bii) As above, but a transparent view is shown to allow easier visualisation of the DNA in general and the flipped C6 in particular. (C) Sequences of *ter* sites (Continued)

FIGURE 1 (Continued)
A–J from *E. coli* MG1655. The *ter* consensus sequence is shown at the top. Base pairs 1–5, 7, and 19–23 show a higher degree of variability and are shaded in a lighter grey. The highly conserved *ter* core (6, 8–18) is highlighted by a darker grey. Tus binding (Toft et al., 2021), *in vivo* fork blocking observed by 2D gel electrophoresis (Duggin and Bell, 2009) and analysis whether a *ter* site is part of an open reading frame (Duggin and Bell, 2009; Goodall et al., 2021) are indicated (see text for further details).

consequences that can arise as a result of fork fusions, we suggest that the strict use of only a single origin per chromosome observed in all bacteria helps to reduce the number of termination events to exactly one, thereby limiting pathologies triggered by fork fusions.

Mechanism of fork arrest by Tus-ter in *Escherichia coli*

How can a single Tus-ter complex be such an efficient block to the progression of replication fork complexes? Functional *ter* sites bound by the monomeric terminator protein Tus block replication in a polar manner. Key to this polar activity is that Tus binding is asymmetric, a feature that has been extensively studied (Neylon et al., 2005; Elshenawy et al., 2015; Pandey et al., 2015; Berghuis et al., 2018). Arrest of an approaching replication fork complex is triggered when the double-stranded DNA immediately adjacent to one face of Tus, called the non-permissive face, is unwound as the fork approaches (Mulcair et al., 2006). The structural change in the opened double helix induces specific contacts between the DNA and Tus that result in the formation of ‘locked complex’, which will block progression of the oncoming replisome with surprising efficiency, a mechanism referred to as a ‘mousetrap’ (Mulcair et al., 2006). Specifically, a base on the leading strand template (C in position 6) flips into a binding site on Tus, guided by nearby residues (Figure 1B and Table 1). This mechanism of ‘clicking into place’ essentially blocks any movement along the DNA by a ratchet-type mechanism, and also slows the dissociation of Tus (Mulcair et al., 2006; Berghuis et al., 2015; Elshenawy et al., 2015; Pandey et al., 2015). This ‘locked’ Tus-ter complex can be generated by unwinding of the double-stranded DNA alone; DnaB helicase is not essential for its formation (Bastia et al., 2008; Berghuis et al., 2015; Elshenawy et al., 2015; Toft et al., 2022). Some stabilization by specific protein–protein interaction between Tus and DnaB has been reported (Mulugu et al., 2001), but to what extent they contribute towards the efficient arrest is not clear. Multiple labs have highlighted key residues which have major roles in the formation of a successful Tus-ter lock (Table 1). Mutants in these residues alter the C6 binding, ssDNA guiding, and slowing the momentum of the approaching replisome.

If individual Tus-ter complexes block replication progression so effectively, why are so many *ter* sites found in the *E. coli* chromosome? It has become increasingly clear that not all sequences identified as *ter* sites block forks. Also, even a *ter* site with a strong blocking activity is not an absolute block. In a study using 2-dimensional neutral-neutral gel electrophoresis analysis, Duggin and Bell were able to show that in wild type cells the vast majority of blocked forks occur at the innermost *ter* sites of the fork trap system. Highest levels were detected at *terC* and *terB*, followed by *terA*. In this study rather mild levels of

TABLE 1 Key amino acid residues of *E. coli* Tus protein and their role in blocking progression of replication fork complexes.

Residue	Importance	Reference
E47	Stabilize 'mousetrap'	Mulugu et al. (2001) and Toft et al. (2021)
E49	Slow approaching fork <i>via</i> helicase interaction	Berghuis et al. (2015)
F140	Holding C6 in place	Mulcair et al. (2006)
H144	Holding C6 in place	Mulcair et al. (2006)
G149	Holding C6 in place	Mulcair et al. (2006)
R198	Slow approaching fork <i>via</i> helicase interaction	Elshenawy et al. (2015)

signal were found for *terD* and *terG* (Figure 1C), and the lowest levels were identified for *terH* and *I*, while *ter* sites *E*, *F* and *J* showed no signal in wild type cells (Duggin and Bell, 2009). In a recent study by the Schaeffer lab, ChIP-Seq was used to identify which *ter* sites are actually occupied by Tus protein. The authors identified *ter* sites *A–E* and *G* (Figure 1C), whereas no Tus occupancy was observed at any of the other *ter* sites (Toft et al., 2021).

These observations fit well with our own data. The replicore arrangement can be significantly skewed by introducing additional ectopic copies of the *oriC* sequence into the chromosome. In a strain with two active replication origins (Wang et al., 2011; Ivanova et al., 2015; Dimude et al., 2018b), one fork reaches Tus-*ter* complexes much earlier than the other. Depending on whether the ectopic origin is located in the left-hand or right-hand replicore, forks get arrested at *terC/B* or *terA/D* (Figure 2A), which can be visualized by using high-resolution marker frequency analysis (MFA) from whole genome sequencing (WGS) (Ivanova et al., 2015; Dimude et al., 2018b; Syeda et al., 2020). These replication profiles, generated by WGS, reveal initiation sites of synthesis, such as *oriC*, as distinct peaks, whereas termination sites form a v-shaped valley, with a replication gradient between initiation and termination sites (Skovgaard et al., 2011; Figure 2Bi). Our MFA analyses confirm that the four innermost *ter* sites *A–D* form a strong block. All four are low points in the replication profile (Figures 2Bii,iii), in line with the idea that individual Tus-*ter* complexes can be overcome occasionally, but the strong 'step' in the replication profile confirms the considerable block that is formed, especially by the innermost *ter* sites *A* and *C/B*.

Additional information comes from the replication profiles of strains that show over-replication of the termination area. Over-replication of the chromosome can be triggered by the absence of certain proteins, such as Tus, RecG or 3' exonucleases, resulting in a peak of synthesis between *ter* sites *A* and *B/C* (Markovitz, 2005; Duggin et al., 2008; Rudolph et al., 2009, 2013; Wendel et al., 2014; Midgley-Smith et al., 2019). Synthesis can proceed beyond *ter* sites *C* and *B* under certain conditions in cells lacking RecG helicase (Figure 2C; Midgley-Smith et al., 2018). The precise molecular mechanism for this regular progression of synthesis beyond *ter* sites *C* and *B* is not clear. However, the ChIP-Seq data from the Schaeffer lab would predict that escape synthesis should proceed past *terF* before getting blocked by *terG* (Toft et al., 2021). This is indeed precisely what we observed, supporting the idea that *terF* is not blocking the progression of forks in normal cells, whereas *terG* can

block ongoing synthesis in wild type cells (Figure 2C). Thus, while the majority of the work of blocking forks is done by three of the innermost *ter* sites *A* and *B/C*, with *terD* clearly being active (Figure 2B) but contributing relatively little, as observed before (Duggin and Bell, 2009), some progressing synthesis can also be blocked by more distal *ter* sites, such as *terG*.

Given that the innermost Tus-*ter* complexes in particular very efficiently block progression of ongoing synthesis, the fusion of replication fork complexes will occur almost exclusively within the innermost termination area. However, this does not answer the question of whether Tus-*ter* complexes are a regular component of termination, or if they are only involved when progression of the two replication fork complexes becomes asynchronous, for example if one of the two complexes is arrested by a DNA lesion, a secondary structure or a protein-DNA complex for any length of time.

Early DNA labeling experiments demonstrated that fork fusion events typically occur close to the numerical midpoint of the chromosome and away from Tus-*ter* complexes (Bouché et al., 1982). This finding is supported by our own MFA-based replication profiles. MFA-based replication profiles use 1 kb windows per individual data point, which means resolution is relatively coarse (Skovgaard et al., 2011; Rudolph et al., 2013). However, within the limits of this resolution, our data demonstrate that the fork fusion point, as determined by LOESS regression, is in precisely the same location in the presence and absence of Tus protein (Rudolph et al., 2013; Dimude et al., 2016). The only change observed is a mild distortion of the marker frequencies in the vicinity of the fusion point (see Dimude et al. (2016) and Goodall et al. (2021) for a direct comparison of the replication profiles in the presence and absence of Tus terminator proteins). In contrast, in cells in which the replicore arrangement is artificially skewed by an additional ectopic origin, the fork fusion point is significantly shifted if the *tus* gene is deleted, as expected (Ivanova et al., 2015; Dimude et al., 2018b; Syeda et al., 2020). These findings suggest that a significant proportion of fork fusion reactions take place in a relatively short stretch between the *dif* chromosome dimer resolution site and *terB*, regardless of whether Tus is present or absent (Rudolph et al., 2013; Ivanova et al., 2015; Dimude et al., 2016). However, the fact that the termination area changes shape in the replication profile of cells lacking Tus demonstrates that Tus-*ter* complexes do contribute to termination on a regular basis (Rudolph et al., 2013; Dimude et al., 2015). Indeed, in a modelling study by Kono and colleagues it was demonstrated that a mixture of Tus-*ter* based and free fusion reactions was shown to result in GC-skew profiles that represented the experimental data most closely (Kono et al., 2012). Additionally, the authors did observe significant differences of the GC-skew profiles in bacteria which do not have a Tus or RTP-based fork trap system (Kono et al., 2012). Taken together, the data suggest that replication often terminates *via* the free fusion of forks, but if a fork trap system is present this also contributes to the termination process on a regular basis.

Fork trap systems in other bacterial species

Galli and co-workers recently analyzed the bacterial species that have a Tus-*ter* fork trap system. They showed that the presence of a replication fork trap in the termination area of the chromosome is a specialized feature of a relatively small group of bacteria (Galli et al.,

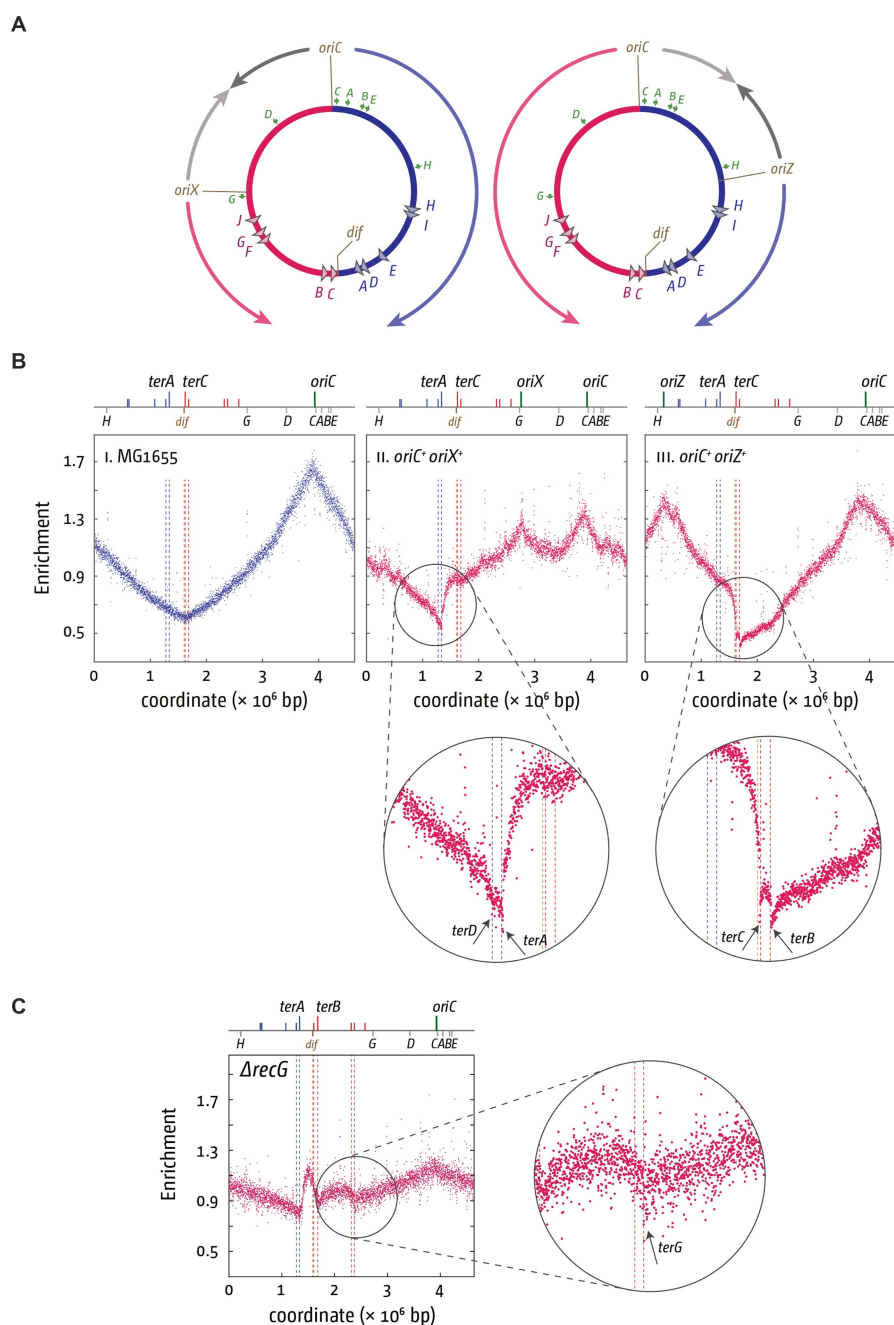


FIGURE 2
 Altered replicore structure in *E. coli* cells with additional ectopic replication origins. **(A)** Schematic representation of *E. coli* chromosomes with additional ectopic replication origins *oriX* (left) and *oriZ* (right), respectively. Positions of *oriC*, *ter* sites as well as the *dif* site and *rrn* operons A–E, G, and H are shown. **(Bi–iii)** Marker frequency analysis (MFA) in MG1655, *oriC*⁺ *oriX*⁺ and *oriC*⁺ *oriZ*⁺ cells. The number of reads (normalized against reads for a stationary phase wild type control) is plotted against the chromosomal location. A schematic representation of the *E. coli* chromosome showing positions of *oriC*, *oriX* and *oriZ* (green lines) and *ter* sites (all above) as well as *dif* and *rrn* operons A–E, G, and H (all below) is shown above the plotted data. The MFA raw data were taken from Ivanova et al. (2015) and Dimude et al. (2018b) and re-plotted to allow axis scale changes, if necessary. The areas where forks are blocked by Tus-*ter* complexes are highlighted by circles and details magnified below to highlight the fork blocking ability of *ter* sites A, B, C, and D. **(C)** Over-replication in the termination area of $\Delta recG$ cells growing in M9 minimal salts with glucose. The area where forks escaping the innermost *ter* sites are blocked by Tus-*ter* complexes are highlighted by a circle, and details magnified to the side to highlight the fork blocking ability of *ter* site G. The MFA raw data were taken from Midgley-Smith et al. (2018) and re-plotted.

2019). This suggests that perhaps the majority of bacterial species do not have a fork trap mechanism. Proteins with homology to Tus can be found in most Enterobacteriales, the Pseudoalteromonas and most Aeromonadales (Galli et al., 2019). As already highlighted, a fork trap

system can be found in *B. subtilis*, but distribution amongst *Bacillus* species is even more restricted and the dissimilarity to Tus-*ter* strongly suggests that its origin is different and has arisen as a result of convergent evolution, highlighting the importance of a fork trap

(Franks et al., 1995; Neylon et al., 2005; Galli et al., 2019). Indeed, the binding mechanisms of the terminator proteins to their *ter* sites are different, with Tus being a polar monomer and the *B. subtilis* terminator protein, RTP, being a dimer that requires a conformational change to arrest forks (Eli Bussiere et al., 1995; Franks et al., 1995; Pai et al., 1996; Griffiths et al., 1998; Wilce et al., 2001). In the *B. subtilis* chromosome, terminator sites are formed *via* two ‘half-sites’, the A and the B site, each bound by an RTP dimer (Lewis et al., 1990). Thus, in total four RTP monomers bind to each *ter* sequence (Lewis et al., 1990). However, binding of an RTP dimer to the A site is much weaker than binding of the second dimer to the B site. Consequently, forks arriving at the B site first will be blocked, while forks arriving at the A site can proceed (Smith and Wake, 1992), which is fundamentally different to the *E. coli* Tus-*ter* system. Interestingly, the transcriptional regulation of the genes encoding for RTP and Tus are achieved through auto-regulation, with a *ter* site being located in the promoter area of the respective genes (Natarajan et al., 1991; Ahn et al., 1993).

The phylogenetic analysis by Galli and co-workers resulted in the interesting hypothesis that the *E. coli* fork trap system might have been domesticated from plasmid-based precursor systems such as in R1 (Figure 3A). The authors highlight that in the Pseudoalteromonadales, *tus* is always located directly adjacent to the origin of the secondary chromosome, which is a mega plasmid (Galli et al., 2019; Figure 3B). Indeed, the GC skew for the secondary chromosome in *Pseudoalteromonas haloplanktis* fits a unidirectional replication mode (Médigue et al., 2005; Xie et al., 2021). A fork trap in close proximity to the origin, together with the unidirectional mode of replication, is indeed very reminiscent of the system found in the R1-type plasmids (Figure 3A; Krabbe et al., 1997).

If the fork trap system was domesticated in this fashion, it is easy to imagine that the unidirectional mode of replication will become rate-limiting for the size of chromosomes, a problem easily solved by a ‘remodelling’ of the chromosome that resulted in the integration of sequences between the origin and the Tus-*ter* complexes (Figure 3C). A prediction from this hypothesis would be that there might be chromosomes with a single *ter* site limiting progression of one of the replichores, and where the *tus* gene is associated with this innermost *ter* site. In a recent study by Toft and colleagues it was indeed highlighted that two distinct fork trap architectures exist (Toft et al., 2021). The fork trap system in *E. coli* is classed as a type II system, because the *ter* site regulating transcription of the *tus* gene is *terB*, which is a secondary rather than the innermost *ter* site (Toft et al., 2021). Outside of the Enterobacteriaceae, another system exists which the authors classified as a type I system. In organisms such as *Edwardsiella tarda* or *Dickeya paradisiaca* a single *ter* site is found to block progression of one replichore, and also acts as a transcriptional repressor of the *tus* gene (Toft et al., 2021, 2022), exactly as predicted. In organisms such as *Edwardsiella tarda* and *Cedecea neteri* the second replichore is also restricted by only a single *ter* site, whereas in organisms such as *Dickeya paradisiaca* two functional *ter* sites are used (Toft et al., 2021, 2022). The authors speculate that the type I system is the ancestral system, whereas the type II system is more divergent (Toft et al., 2021, 2022), which seems overall to fit well with the notion of a plasmid-derived system (Figure 3; Galli et al., 2019).

However, while the classification into a type I and II system is helpful to some degree, the systems are likely to be more similar than they appear upon casual inspection. There is no doubt that the number of innermost *ter* sites differ across species, together with the

association and relative location of the *tus* genes. But the outer *ter* sites *F–J* in *E. coli* are all part of open reading frames, which means that, regardless of whether it ever was a functioning *ter* site or whether the sequence similarity is coincidence, the sequence will be conserved as part of the maintenance of the genes they are in. Both arguments are not mutually exclusive, but the ChIP-Seq data from the Schaeffer lab highlight that the occupancy of *ter* sites *F* and *H–J* is low (Toft et al., 2021). Indeed, when we analyzed the sequence conservation of all *ter* sites across all phylogenetic groups in *E. coli*, we found a revealing trend: for the innermost *ter* sites *A–D*, which clearly have a role in blocking forks (Duggin and Bell, 2009; Toft et al., 2021), strict conservation was observed for all bases that make contact with Tus. However, especially at bases 1–5 and 7 numerous variations were found, which are unlikely to affect Tus binding (Goodall et al., 2021). Interestingly, the same pattern was observed for *terG* (Goodall et al., 2021), the one *ter* site which is not part of the inner fork trap, but for which Tus binding and significant fork blocking activity was demonstrated (Duggin and Bell, 2009; Midgley-Smith et al., 2018; Toft et al., 2021). For *ter* sites *E*, *F* and *H–J* far fewer sequence variations were observed, a trend that extends beyond the 23 bp *ter* sequence (Goodall et al., 2021), in line with the idea that the sequence conservation might be driven to a large extent by the ORF they are in Table 2.

In this analysis *terE* stands out, because it is not part of an ORF (Table 2), but is still highly conserved (Goodall et al., 2021). *terE* is located within the *efeUOB* operon, which, in enterohaemorrhagic *E. coli* O157:H7, encodes an iron transport system (Cao et al., 2007). *terE* is located between *efeU*, a pseudo-gene in *E. coli* K12 (Grosse et al., 2006), and *efeO*. In our sequence analysis across all *E. coli* phylogroups we did not find any variations of *terE*, nor of the wider region around the actual *terE* sequence (Goodall et al., 2021). This conservation makes it tempting to speculate that it is located in a sequence environment with an important regulatory function, even though we do not know what this function is. In contrast, when we analyzed *terY*, a pseudo *ter* site that neither acts as a part of the fork trap nor is it located within an ORF (Table 2), we observed significant sequence variations across the phylogenetic groups, as well as changes in its relative location, in line with the idea that this sequence snippet is subjected to unselective evolutionary drift (Goodall et al., 2021).

Taken together, the data suggest that we can simplify the fork trap system in *E. coli* and related bacteria by focusing on the innermost *ter* sites *A–D* as those being active in termination. The main difference between the type I and type II systems remains whether the border of the fork trap is defined by a single *ter* site associated with the *tus* gene (type I), or whether a cluster of *ter* sites is used, with *tus* associated with one of the secondary *ter* sites (type II) (Toft et al., 2022). All of the other *ter* sites in *E. coli* are likely to be still present because of selection pressures unrelated to termination issues.

Fork trap systems can pose problems for DNA replication

The defined architecture of the bacterial chromosome, where a single origin results in each replichore being replicated by a single replication fork complex, dictates that any restriction to fork movement will become problematic if a fork is held up unexpectedly, for example

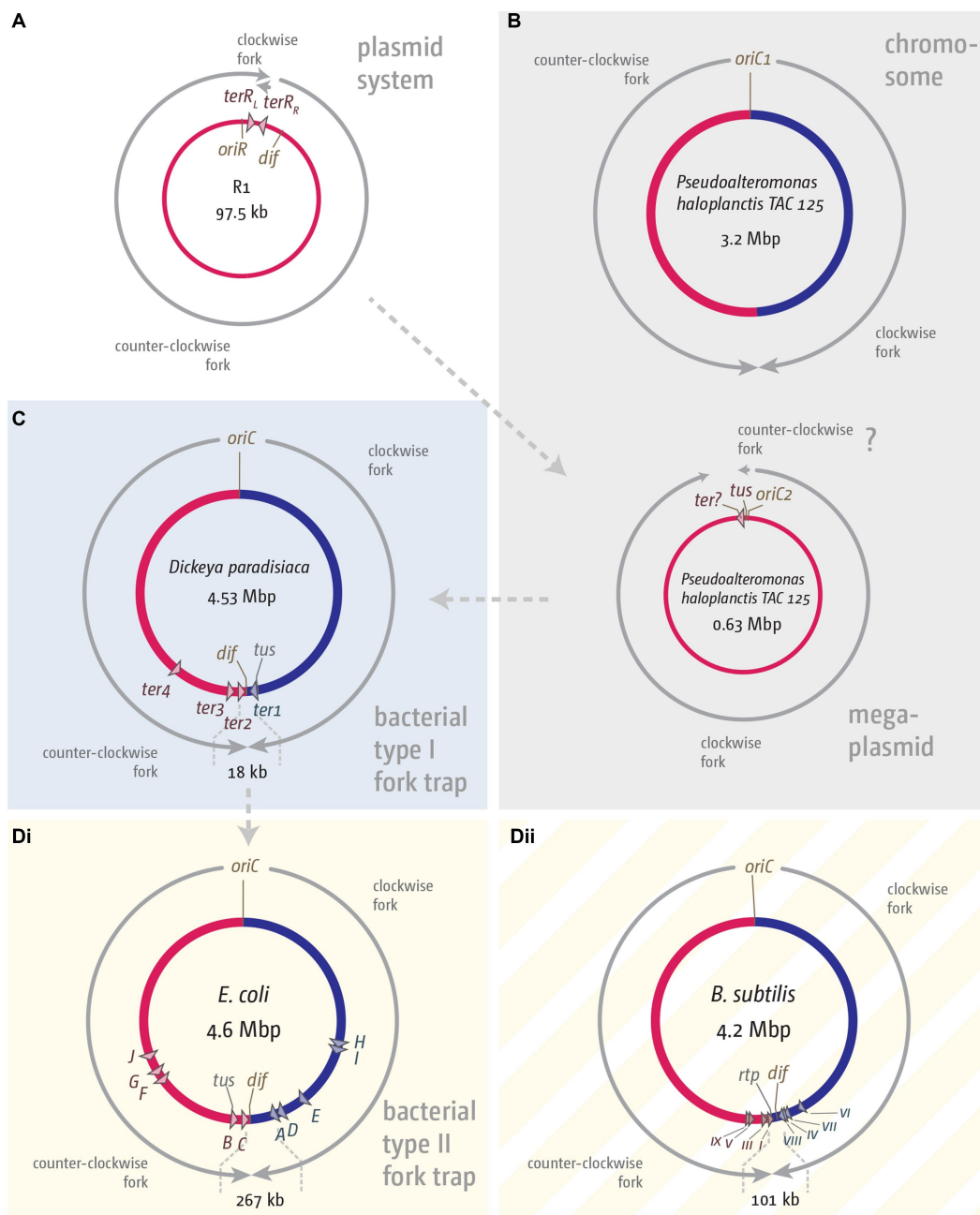


FIGURE 3 Comparison of fork trap systems in various plasmids and bacterial chromosomes. **(A)** Replication dynamics and fork trap system in the plasmid R1. **(B)** Replication and fork trap features of the primary and secondary chromosome in *Pseudoalteromonas haloplanctis* TAC 125. The secondary chromosome contains a *tus* gene, which is located next to the origin of replication, *oriC2*. The presence of a *ter* site is only implicated and therefore marked with a question mark. **(C)** Type I fork trap system in *Dickeya paradisiaca*, as described in Toft et al. (2022). **(D)** Type II fork trap system in *E. coli* **(Di)** and the fork trap system in *Bacillus subtilis* **(Dii)**. See main text for further details.

at a small DNA lesion or a protein-DNA complex (Lang and Merrikh, 2018; Mariani, 2018; Bianco, 2020; Linke et al., 2021). Any stalled fork has to be reactivated to complete chromosome replication, as the only other fork available is blocked by Tus-ter complexes, a clear disparity to the concept of dormant origins in eukaryotic cells (Blow et al., 2011; Shima and Pederson, 2017). If fork reactivation cannot be achieved and the other fork is arrested by the multiple Tus-ter complexes in its way, the chromosome will remain only partially replicated, with potentially lethal consequences for the cell.

The difficulties caused by this scenario can be shown experimentally in cells in which the replicore arrangement is distorted. This was done in *B. subtilis* in which a *ter* site was integrated in blocking orientation into an ectopic location of the chromosome, on the edge of the replication fork trap system. Forks were blocked for a significant period of time, causing significant morphological changes of the affected cells (Franks and Wake, 1996). Indeed, similar experiments were done in *E. coli* by the Michel lab, showing that arrested forks only can be reactivated if a second round of replication

TABLE 2 Analysis of various parameters of all predicted *ter* sites in the *E. coli* chromosome.

Site	Chromosomal location [Mbp]	Within ORF	Sequence conservation	Fork block	References
<i>terA</i>	1.34	mixed	1 SNP/23 bp	Yes	Duggin and Bell (2009), Ivanova et al. (2015), Dimude et al. (2018b), and Toft et al. (2021)
<i>terB</i>	1.68	No	1–2 SNPs/23 bp	Yes	
<i>terC</i>	1.61	No	1–2 SNPs/23 bp	Yes	
<i>terD</i>	1.28	No	1–2 SNPs/23 bp	Yes	
<i>terE</i>	1.08	No	0 SNP/23 bp	Not observed	Duggin and Bell (2009)
<i>terF</i>	2.32	Yes	1 SNP/23 bp	No	Duggin and Bell (2009), Midgley-Smith et al. (2018), and Toft et al. (2021)
<i>terG</i>	2.38	Yes	1–2 SNPs/23 bp	Yes	Duggin and Bell (2009), Midgley-Smith et al. (2018), and Toft et al. (2021)
<i>terH</i>	0.60	Yes	0 SNP/23 bp	No	Duggin and Bell (2009) and Toft et al. (2021)
<i>terI</i>	0.625	Yes	1 SNP/23 bp	No	Duggin and Bell (2009) and Toft et al. (2021)
<i>terJ</i>	2.58	Yes	1 SNP/23 bp	No	Duggin and Bell (2009) and Toft et al. (2021)
<i>terK</i>	2.28	Yes	nd ^a	No	Duggin and Bell (2009)
<i>terL</i>	2.88	Yes	nd ^a	No	Duggin and Bell (2009)
<i>terY</i>	2.87	No	5 SNPs/23 bp	No	Duggin and Bell (2009)
<i>terZ</i>	3.44	Yes	nd ^a	No	Duggin and Bell (2009)

^aWe did not systematically determine the sequence variations across the *E. coli* phylogroups for *ter* sites K, L, and Z.

reaches the arrested primary fork, resulting in the generation of linear DNA molecules that engage in homologous recombination and allow reactivation of forks. If recombination is inactivated, the presence of an ectopic *ter* site proved lethal (Bidnenko et al., 2002, 2006).

In our own work we used strains where, instead of using ectopic *ter* sequences, the origin was moved 1 Mbp into an ectopic position. In this scenario one of the two forks initiated will duplicate $\frac{1}{4}$ of the chromosome before being arrested at Tus-*ter* complexes, while the other fork has to duplicate the remaining $\frac{3}{4}$ of the chromosome. In addition, this fork has to replicate $\frac{1}{4}$ of the chromosome in an orientation opposite to normal. The doubling time of such a construct was approximately 40 min, twice that of the 20 min normally observed for wild type cells (Ivanova et al., 2015). Inactivation of the replication fork trap by the deletion of *tus* reduced the doubling time to ~30 min (Ivanova et al., 2015). Together, the findings in these experimental systems strongly highlight the impact the replication fork trap has to impede the movement of replisomes (Franks and Wake, 1996; Bidnenko et al., 2002; Ivanova et al., 2015).

However, even if one fork is blocked at an obstacle only for a limited period of time, the replication fork trap still poses a threat to the completion of DNA synthesis. At fork speeds of 700–1,000 nt/s (Baker and Bell, 1998; Pham et al., 2013) even a short delay of one of the forks will cause the second to get arrested at a Tus-*ter* complex. This means that, upon reactivation, the temporarily arrested fork will eventually reach a replication fork complex that is stably arrested at a Tus-*ter* complex. While the arrest of a single replisome by Tus-*ter* complexes has received considerable attention, and a substantial number of studies have looked at the effects of Tus-*ter* on the progression of reconstituted replication fork complexes *in vitro* (Khatri et al., 1989; Lee et al., 1989; Hill and Marians, 1990; MacAllister et al., 1990; Gottlieb et al., 1992; Hiasa and Marians, 1994; Neylon et al., 2000), the fusion reaction of a freely moving fork with another stably arrested at a Tus-*ter* complex has not really been studied, even

though this is likely to be physiologically very relevant. We were recently able to reconstitute this specific reaction *in vitro*. We used a plasmid template with an *oriC* sequence which, upon adding all the purified *E. coli* replisome components, together with the DnaA and DnaC initiator proteins, allows the formation of fully functional replication fork complexes (Jameson et al., 2021). The plasmid substrate contains *terB*, which, upon addition of Tus, will block progression of one of the two established forks (Figure 4A). The second fork moving in the opposite direction can be blocked at an array of 22 copies of the *lacO* sequence if the *lac* repressor protein LacI is added. The block is temporary, because addition of IPTG allows release of the block and continuation of synthesis until the second fork reaches the Tus-*ter* complex from the permissive side (Figure 4A). This precisely mimics the scenario of a moving fork running into another stably arrested at a Tus-*ter* complex (Jameson et al., 2021).

Termination mapping analysis revealed that in this scenario the template also remains under-replicated. We observed a gap of between 15 and 24 bp, which matches the footprint of Tus (Figure 4B; Jameson et al., 2021). Thus, it is very tempting to speculate that, as a result of this particular type of fusion reaction, Tus remains stably bound, sandwiched between two replication fork complexes (Figure 4C; Jameson et al., 2021). We currently do not know how this complex is resolved *in vivo*. It seems likely that a helicase needs to be involved to remove the bound Tus protein and facilitate progression of at least one of the two forks to facilitate completion of DNA synthesis (Figure 4C). However, we do not currently know which helicase might be involved in this reaction (Jameson et al., 2021).

Nevertheless, both scenarios highlight that, within the defined architecture of the *E. coli* chromosome, a fork trap poses a risk that can prove fatal. We therefore wanted to investigate whether we could find a fork trap system that was inactivated by point mutations in specific *E. coli* strains. *E. coli* can be found in the gastrointestinal tract of a wide variety of warm-blooded animals (Stoppe et al., 2017). These

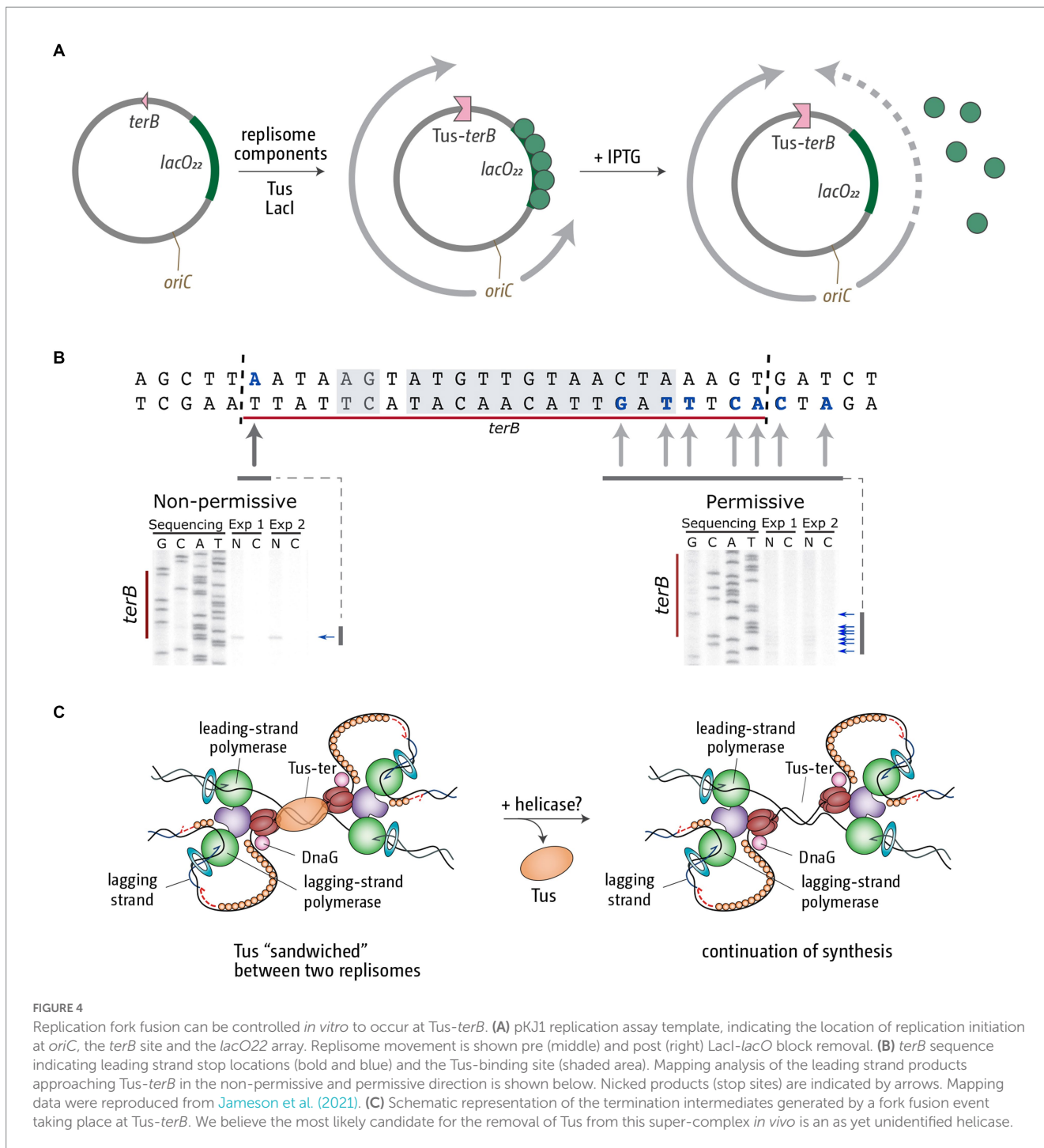


FIGURE 4
Replication fork fusion can be controlled *in vitro* to occur at Tus-*terB*. (A) pKJ1 replication assay template, indicating the location of replication initiation at *oriC*, the *terB* site and the *lacO₂₂* array. Replisome movement is shown pre (middle) and post (right) Lacl-*lacO* block removal. (B) *terB* sequence indicating leading strand stop locations (bold and blue) and the Tus-binding site (shaded area). Mapping analysis of the leading strand products approaching Tus-*terB* in the non-permissive and permissive direction is shown below. Nicked products (stop sites) are indicated by arrows. Mapping data were reproduced from Jameson et al. (2021). (C) Schematic representation of the termination intermediates generated by a fork fusion event taking place at Tus-*terB*. We believe the most likely candidate for the removal of Tus from this super-complex *in vivo* is an as yet unidentified helicase.

populations are shaped by factors specific to the host as well as the environment, resulting in a high degree of diversity. This diversity is reflected in a core genome that contains approximately 2,200 genes, while the pan-genome contains more than 13,000 genes (Rasko et al., 2008). However, despite this variability, our sequence analysis did not reveal even a single example of a phylogenetic group where the replication fork trap appears to be non-functional (Goodall et al., 2021). Thus, while a fork trap clearly is not essential, and a wide variety of bacterial species appear not to utilize a fork trap (Galli et al., 2019), it appears that, once acquired, such a fork trap system is strictly maintained (Neylon et al., 2005; Goodall et al., 2021; Toft et al., 2022). Together with the fork trap system in *B. subtilis*, which has a similar

functionality but has likely arisen by convergent evolution (Franks et al., 1995; Neylon et al., 2005), these findings suggest that a fork trap system is involved in mitigating a problem important enough to selectively maintain the fork trap system.

Are replication-transcription conflicts responsible for the evolution of fork traps?

One proposed role for the fork trap is maintaining the co-directionality of transcription and replication (Brewer, 1988). Head-on collisions of replication and transcription complexes pose a

challenge to cells (Rudolph et al., 2007; Kim and Jinks-Robertson, 2012; McGlynn et al., 2012; Merrikh et al., 2012; Lang and Merrikh, 2018), a challenge that becomes especially significant if collisions occur at highly-transcribed genes such as the operons for ribosomal RNA (Wang et al., 2007; Boubakri et al., 2010; Srivatsan et al., 2010; De Septenville et al., 2012; Ivanova et al., 2015; Dimude et al., 2018a; Hawkins et al., 2019). Especially highly transcribed genes, such as *rrn* operons and genes encoding ribosomal proteins, are transcribed co-directionally with the direction of replication (Brewer, 1988; McLean et al., 1998). If a fork would proceed beyond the termination area, perhaps because the other is held up at an obstacle, the proceeding fork will now encounter these genomic areas in the dangerous head-on orientation, which is normally prevented by a fork trap.

Is this likely to be the driver behind the strict maintenance of a fork trap area? In *E. coli* all *rrn* operons and the majority of genes encoding ribosomal proteins are located in the origin-proximal half of the chromosome (McLean et al., 1998; Dimude et al., 2016). Forks proceeding from the termination area in the wrong orientation would have to duplicate 1 Mbp and more before they would even reach these highly transcribed areas. At least under laboratory conditions, and within the resolution limit of the MFA used, the fork fusion point is surprisingly consistent (Rudolph et al., 2013; Dimude et al., 2016; Goodall et al., 2021), which is perfectly in line with the relatively small percentages of stalled forks detected by 2D gel electrophoresis (Duggin and Bell, 2009). Together, this suggests that long delays of a single fork are a rare event.

For the rest of the *E. coli* chromosome co-directionality is approximately 55% (McLean et al., 1998). However, this does not necessarily mean that directionality is random. Data from Merrikh and colleagues have shown that head-on transcription-replication conflicts increase the incidence of mutagenesis that can result in adaptive mutations, which actually increase cell survival (Merrikh et al., 2012, 2016; Merrikh, 2017; Merrikh and Merrikh, 2018). This arises from the fact that many stress-induced genes are encoded on the lagging strand, thereby triggering head-on collisions with transcription machinery and, as a result, location-specific mutagenesis (Schroeder et al., 2016, 2020). Thus, while co-directionality reduces conflicts and for this reason is favored for highly transcribed genes, head-on collisions might be advantageous during times of stress, as they are useful for adaptive mutagenesis (Lang and Merrikh, 2018; Schroeder et al., 2020). Still, these effects are likely to be more subtle than the consequences of head-on collisions at highly-transcribed locations.

To gain at least some insight into whether forks escaping the termination area are causing any systematic problems before reaching the highly-transcribed *rrn* operons we used cells which contain ectopic replication origins in addition to *oriC*, as described above, and in which the fork trap is inactivated by the deletion of the *tus* gene. In Δ *tus* cells carrying an ectopic replication origin called *oriZ* roughly half way into the clockwise replicore, the fork fusion point is shifted significantly into the counter-clockwise replicore, with one fork escaping the termination area and duplicating roughly 500 kb before fusing with the opposing fork (Ivanova et al., 2015). The mirrored situation takes place in Δ *tus* cells carrying an ectopic replication origin called *oriX* roughly half way into the counter-clockwise replicore (Dimude et al., 2018b). Our marker frequency analysis did not reveal any systematic delays of the forks escaping the termination

area, very much in contrast to the situation observed at *rrn* operons (Ivanova et al., 2015; Dimude et al., 2018a,b; Hawkins et al., 2019). Thus, we currently do not have any data supporting the idea that avoiding the negative consequences of head-on replication-transcription clashes in the origins-distal half of the chromosome is contributing in a major way to the evolutionary maintenance of the replication fork trap, even though control of directionality imposed by the fork trap almost certainly will be an added bonus. It would be interesting to analyze mutation rates and spectrums in the area replicated opposite to normal to also investigate the potentially beneficial effects of replication-transcription conflicts in this area of the chromosome.

Making sense of the replication fork trap

A role in preventing over-replication

If we assume that the fork trap system was derived from an ancestral system that came from plasmids, it is important to consider what is known about the physiological role of such plasmid-born fork trap systems. As described before, for R1 plasmid replication the fork trap mechanism enforces unidirectional DNA replication. Two *ter* sites, *terR_L* and *terR_R*, are located between the minimal origin sequence and the start site for leading strand synthesis (Hill et al., 1988b). DNA replication proceeding counter-clockwise is almost immediately blocked by *terR_L*, whereas the clockwise fork duplicates most of the plasmid DNA until it is blocked at *terR_R* (Krabbe et al., 1997). Interestingly, for R1 replication the absence of Tus protein was shown to result in the accumulation of complex branched DNA structures, plasmid multimers and rolling circle replication intermediates, which interfered with successful plasmid segregation (Krabbe et al., 1997). These findings strongly suggest that the replication fork complex, upon encountering the already replicated part of the plasmid, continues to unwind the already generated nascent section if it is not arrested by a Tus-*ter* complex. The resulting intermediates then serve as substrates for the continuation of replication in various ways (Krabbe et al., 1997). This idea is supported by biochemical studies showing that Tus-*ter* complexes forming a fork trap can prevent over-replication of a circular plasmid substrate, both by a traditional *in vitro* study (Hiasa and Marians, 1994) and the recently developed elegant “replication chain reaction” (Hasebe et al., 2018), lending support to a function of the bacterial replication fork trap in preventing over-replication and, as a result, segregation difficulties, which would pose a strong selection pressure.

Is there any indication that the fork trap might have a similar function for the *E. coli* chromosome? It was indeed reported that the deletion of *tus* resulted in low levels of chromosomal over-replication, an effect significantly exacerbated by specific point mutants in the *polA* gene, which encodes for DNA polymerase I (Markovitz, 2005), a polymerase involved in maintenance and repair processes, such as Okazaki fragment maturation and various DNA repair pathways (Kurth and O'Donnell, 2009). We were able to confirm over-replication of the termination area in a strain with a *polA2* point mutation (Midgley-Smith et al., 2018). These data are in line with the idea that the fork trap, together with specific proteins such as polymerase I, is involved in bringing DNA replication to a successful conclusion (Markovitz, 2005; Duggin et al., 2008; Midgley-Smith et al., 2018; Rudolph et al., 2019). Similarly, absence of the terminator

protein RTP in *B. subtilis* results in an increased formation of chromosomal dimers, which could arise from a related mechanism (Lemon et al., 2001).

What might be the molecular structures that arise as part of termination? Systematic analysis of a number of mutants that show over-replication of the termination area have given important insights. Significant levels of over-replication of the termination area were observed in cells lacking RecG helicase (Rudolph et al., 2009, 2013; Wendel et al., 2014; Dimude et al., 2015), an effect that is completely suppressed by point mutations inactivating the helicase activity of the main replication restart protein PriA (Rudolph et al., 2013; Dimude et al., 2015). Even more specifically, the over-replication is suppressed in a PriA point mutation called *srgA1*, encoding PriA[L557P], a PriA protein with a very specific change of the substrate activity. It unwinds a replication fork with both a leading and a lagging strand at the branch point as efficiently as wild type PriA, but it has lost the ability to unwind a fork in which the leading strand is missing (Gregg et al.,

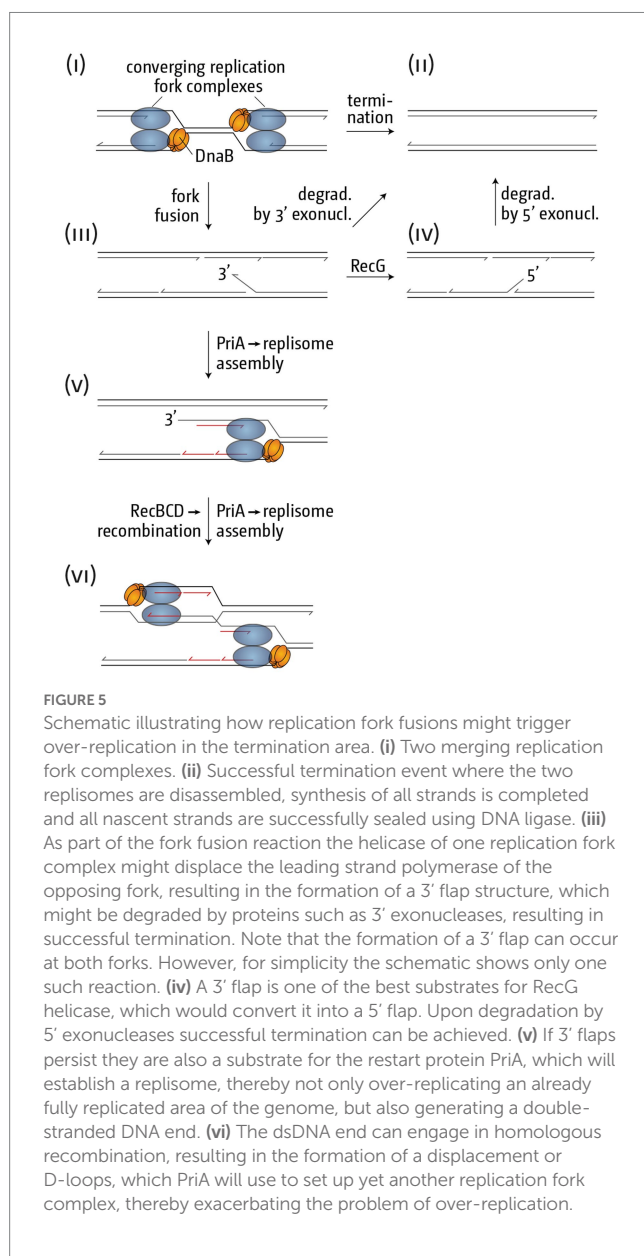
2002), which is, effectively, a 3' flap structure. This result strongly suggests that a 3' flap-like structure is involved in triggering the observed over-replication, and we hypothesized that it arises if, as part of a fork fusion reaction (Figures 5i,ii), the helicase of one replication fork complex displaces the leading strand polymerase of the opposing fork (Figure 5iii). Both template and primer are held in the active center of the replicative polymerase under tension (Joyce and Steitz, 1995; Baker and Bell, 1998; Xu and Dixon, 2018), and a 'nudge' of the leading strand polymerase by the DnaB helicase of the opposing fork might be enough to temporarily melt primer and template.

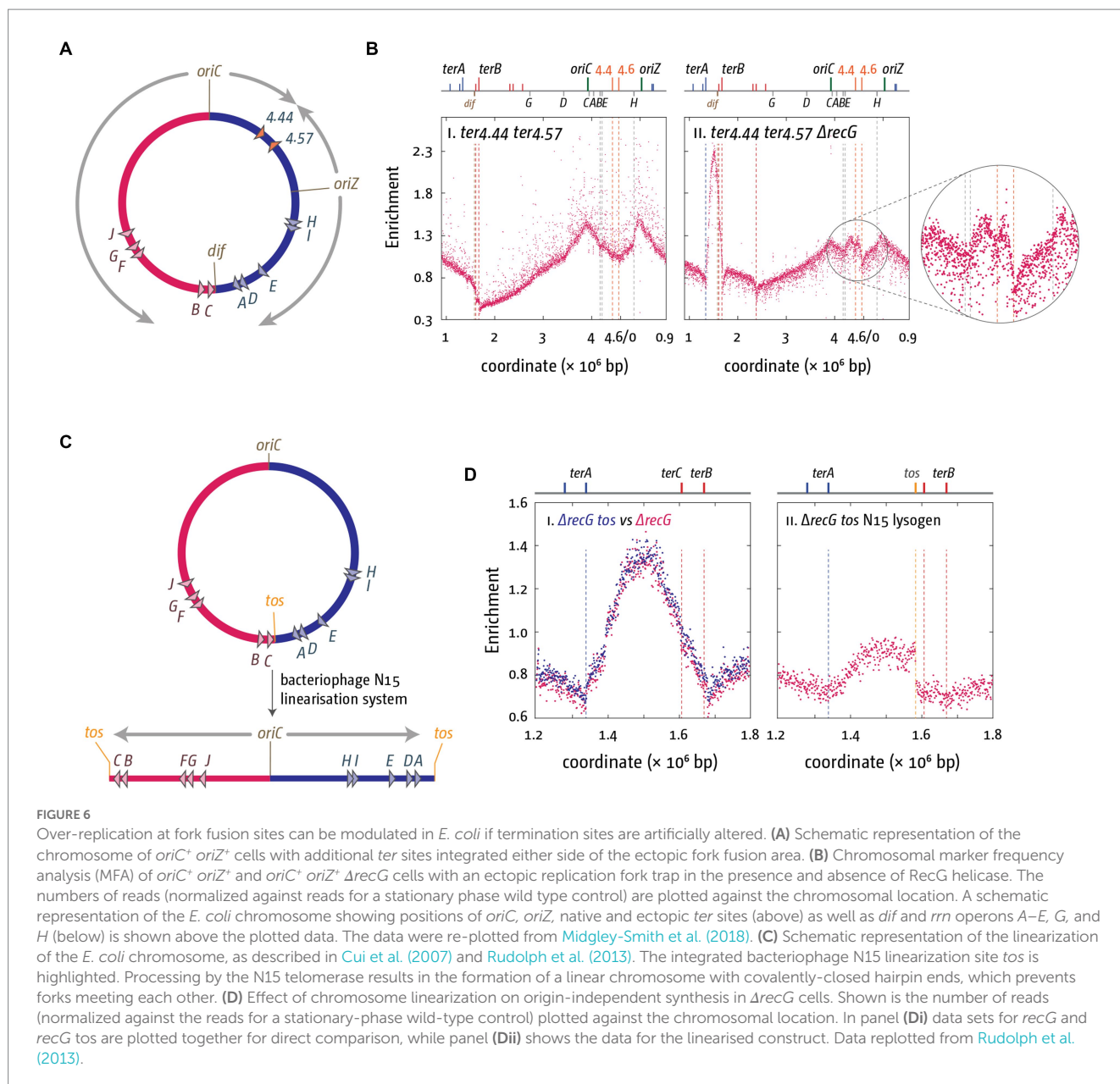
RecG helicase shows a particularly high affinity for 3' flaps and processes these by converting them into a 5' flap structure (Figure 5iv; McGlynn and Lloyd, 2001; Tanaka and Masai, 2006). In addition, 3' flaps will also be degraded by 3' single-stranded exonucleases, and we have shown that the absence of multiple 3' exonucleases in particular results in high levels of over-replication in the termination area (Rudolph et al., 2010, 2013; Midgley-Smith et al., 2019). Thus, we hypothesize that the absence of either RecG or 3' exonucleases results in the persistence of 3' flap-like structures, a substrate for PriA, even though the affinity of PriA for a 3' flap is lower than that of RecG (Tanaka and Masai, 2006). PriA processing will result in the assembly of a new replication fork, the progression of which will not only result in chromosomal over-replication, but also the formation of a double-stranded DNA end (Figure 5v). dsDNA ends will be rapidly processed by the homologous recombination proteins RecBCD and RecA, leading to the formation of a displacement loop (D-loop) (Rudolph et al., 2009, 2013, 2019; Dimude et al., 2016; Midgley-Smith et al., 2019). D-loops are yet another substrate for PriA, which means an opportunity for the formation of more replication forks, thus setting up a positive feedback loop of over-replication (Figure 5vi).

What data support the idea that over-replication is triggered at termination inter mediates generated by freely-fusing forks, and not at structures such as replisomes blocked by Tus-*ter* complexes? The latter cannot be the case, because over-replication was initially found in cells lacking Tus protein (Markovitz, 2005) and several recent studies have confirmed the presence of over-replication in Δ tus cells (Rudolph et al., 2013; Dimude et al., 2015; Midgley-Smith et al., 2018, 2019). Thus, the synthesis observed is triggered independently of the fork trap system, very likely directly at fusing replication fork complexes.

In addition, in a recent analysis we were able to show that over-replication can be triggered in the absence of RecG in defined ectopic chromosomal areas if forks are artificially forced to fuse in these areas (Midgley-Smith et al., 2018). Again, we used a strain that harbors an ectopic replication origin, *oriZ*, half-way into the right-hand replichore (Wang et al., 2011; Ivanova et al., 2015). Forks coming from *oriC* and *oriZ* fuse approximately at position 4.5 Mbp of the chromosome (Ivanova et al., 2015), and we artificially engineered an ectopic fork trap in this location, with *ter* sequences inserted at positions 4.4 and 4.6 Mbp (Figure 6A). No over-replication was observed in this location in *oriC⁺ oriZ⁺* cells in the presence of RecG, but in its absence a clear peak of over-replication was observed (Figure 6B; Midgley-Smith et al., 2018).

We also investigated what happens if replication forks are prevented from fusing. The *E. coli* chromosome can be stably linearized using the bacteriophage N15 linearization system. Cui and colleagues successfully integrated the *tos* linearization sequence into the termination area of the chromosome (Cui et al., 2007). Expression





of the bacteriophage N15 telomerase resulted in the processing of the *tos* linearization sequence into two hairpin ends, which will prevent forks from meeting each other (Figure 6C; Cui et al., 2007; Rudolph et al., 2013). If fork fusion reactions are responsible for the observed over-replication, chromosome linearization should result in a significant reduction of the over-replication. This was indeed what we observed. Over-replication was significantly reduced and entirely depended on forks coming from *oriC* (Figure 6D; Rudolph et al., 2013; Dimude et al., 2015). Taken together both findings strongly support the idea that the over-replication observed in the termination area is triggered by the fusion of replication fork complexes. They are of particular importance in the light of a recent elegant study by Raghunathan and co-workers showing that in cells lacking the DNA adenine methylase, Dam, a peak of over-replication is observed in the termination area, and cells are capable of growing in the absence of a

functional origin (Raghunathan et al., 2019). The authors postulate that an increased number of recombination events in all chromosomal areas triggers significant levels of recombination-dependent replication, which also results in over-replication of the termination area and, because of the fork trap, results in a peak in the marker frequency analysis (Raghunathan et al., 2019). Indeed, a role for RecG in controlling recombination-dependent replication was reported before (Azeroglu et al., 2016; Azeroglu and Leach, 2017), which might explain the small peak of synthesis remaining in *ΔrecG* cells in which the chromosome is linearized (Figure 6D). However, as recombination-dependent synthesis is triggered independently of fork fusion events, the reduction of over-replication in *ΔrecG* cells with a linear chromosome strongly suggests that it is not responsible for the significant levels of synthesis observed in the termination area in cells lacking RecG helicase.

Thus, research by our own and other labs shows that resolving the observed over-replication requires a surprising number of proteins, including RecG helicase, DNA polymerase I, the exonucleases ExoI, ExoVII, SbcCD and RecJ, the homologous recombination proteins RecBC, RecD and RuvABC and others (Markovitz, 2005; Rudolph et al., 2013; Wendel et al., 2014, 2018; Dimude et al., 2015, 2018a; Sinha et al., 2017, 2018; Midgley-Smith et al., 2018, 2019). In addition, our recent *in vitro* results suggest that more proteins are yet to be discovered (Jameson et al., 2021). As many of these intermediates arise as a direct result of fork fusions, it is tempting to speculate that they can arise generally as part of termination in bacterial DNA replication, and indeed many of the above proteins are well conserved in prokaryotes (and some beyond). Thus, the necessary proteins exist to bring termination to a successful conclusion, regardless of the presence or absence of a fork trap mechanism. However, a fork trap, once acquired, seems to add an additional layer of protection.

What are the particular benefits of a fork trap system? Bacterial cells go to great lengths to ensure that exactly one complete copy of the chromosome is generated per cell cycle (Boye et al., 2000; Skarstad and Katayama, 2013). However, if the over-replication triggered at termination intermediates is allowed to proceed, it results in uncontrolled over-replication of the chromosome, which can undermine the regulatory steps employed to control genome duplication. In cells lacking both RecG and Tus the over-replication that is triggered in the termination area can proceed into both replichores, eventually leading to dangerous head-on collisions with transcribing RNA polymerase complexes at highly transcribed genes such as *rrn* operons, as described above. Such collisions can be alleviated by so-called *rpo** point mutations, point mutations in genes for some of the RNA polymerase subunits which destabilize ternary RNA polymerase complexes (Trautinger et al., 2005; Rudolph et al., 2007). If an *rpo** mutation is introduced in cells lacking RecG and Tus, the synthesis in these cells is robust enough to allow cell growth and division even if the entire native origin is deleted (Rudolph et al., 2013; Dimude et al., 2015). Inhibition of *oriC* firing in $\Delta recG \Delta tus rpo^*$ cells revealed a replication profile that was inverted, with highest levels of synthesis in the termination area where forks normally fuse, while lowest levels were observed in the area around where *oriC* normally is located (Rudolph et al., 2013; Dimude et al., 2015). In contrast, in cells with a fork trap such over-replication is blocked by Tus-*ter* complexes (Rudolph et al., 2013; Dimude et al., 2015).

One danger of replication fork complexes escaping the termination area is that these forks will fuse somewhere in the replichore with forks coming from *oriC*, which poses the risk of repeating the problem (Figure 7i). In contrast, based on our genetics data we have postulated before that fork fusion events at a Tus-*ter* complex might be less prone to triggering over-replication (Midgley-Smith et al., 2019), even though additional proteins might be required to resolve replisome-Tus-*ter*-replisome super-complexes (Figure 4; Jameson et al., 2021). Thus, if replication fork complexes are stably arrested at Tus-*ter* complexes until the next round of synthesis arrives from *oriC*, the resolution of these complexes might result in successful termination (Figure 7ii). In addition, there is evidence of processing by several nucleases in the termination area, including RecBCD and SbcCD (Rudolph et al., 2013; Courcelle et al., 2015; Azeroglu et al., 2016; Sinha et al., 2017, 2018; Dimude et al., 2018a). The molecular details are complex and will depend on the precise intermediates that

are being formed, but if over-replication is blocked relatively quickly, degradation could contribute to simply resolving that situation and achieve successful termination (Figure 7ii), a situation that is much harder to achieve if over-replication is allowed to proceed into the replichore in a direction opposite to normal.

Fork trap systems have multiple benefits

An additional benefit of a fork trap system was recently observed by Hamilton and colleagues. They measured DNA synthesis induced by χ sites, which switch the functionality of RecBCD from dsDNA degradation to the formation of a 3' ssDNA overhang that will be bound by RecA and which can engage in recombination-dependent replication. By using a plasmid-based system the authors were able to show that the addition of *ter* sites successfully limited the amount of replication induced by χ sites, which helped to improve plasmid stability (Hamilton et al., 2023).

Furthermore, any process that involves processive translocation of large molecular machines along DNA will trigger superhelical stress (Chedin and Benham, 2020). This will be particularly problematic if the overall number of replication forks is increased by over-replication forks escaping the termination area. Superhelical stress can result in increased R-loop formation, as R-loops can help to absorb negative superhelicity (Chedin and Benham, 2020). However, increased levels of R-loops can also threaten genomic integrity (Crossley et al., 2019).

In addition, processing of fork fusion intermediates by homologous recombination proteins will increase the frequency of recombination events in the termination area, and elevated levels of recombination were indeed reported for this area in *E. coli* (Horiuchi et al., 1994). Aspects such as over-replication of already replicated DNA, increased levels of R-loops and increased recombination frequencies have all been found to contribute to genomic instability (Finkel et al., 2007; Blow and Gillespie, 2008; Alexander and Orr-Weaver, 2016; Tomasetti et al., 2017; Crossley et al., 2019), highlighting the benefits of a replication fork trap system and adding to the explanation of why fork trap systems, once acquired, are so consistently maintained.

Another aspect where the fork trap area might play an important role is the chromosome dimer resolution site *dif*. Chromosomal dimers can form as a consequence of an odd number of recombination events. FtsK translocase, directed by FtsK-Orienting Polar Sequences (KOPS) towards the termination area and *dif*, controls a site-specific recombination system that resolves chromosomal dimers, which involves the site-specific recombinase XerCD and the *dif* site (Bigot, 2005; Levy et al., 2005; Barre, 2007; Sherratt et al., 2010). In our analysis of the different *E. coli* phylogroups we found that *dif* is consistently located between the innermost *ter* sites A and C (Figure 8). This is also the case for a variety of other bacteria with a fork trap, including *Salmonella enterica*, *Klebsiella variicola* and *Klebsiella pneumoniae*, *Edwardsiella tarda*, *Cedecea neteri* and *Dickeya paradisiaca* (Goodall et al., 2021; Toft et al., 2022). It is likely that this defined location of the *dif* site reflects biological importance. For the successful resolution of chromosomal dimers exactly two *dif* sites of a dimer have to be brought together, followed by XerCD processing (Castillo et al., 2017). If more than two *dif* sites are present, for example because one stretch containing a *dif* site is duplicated via termination-induced over-replication, this will be problematic, because a recombination event between the wrong *dif* sites will not resolve the chromosomal dimer (Figure 7iii; Goodall et al., 2021).

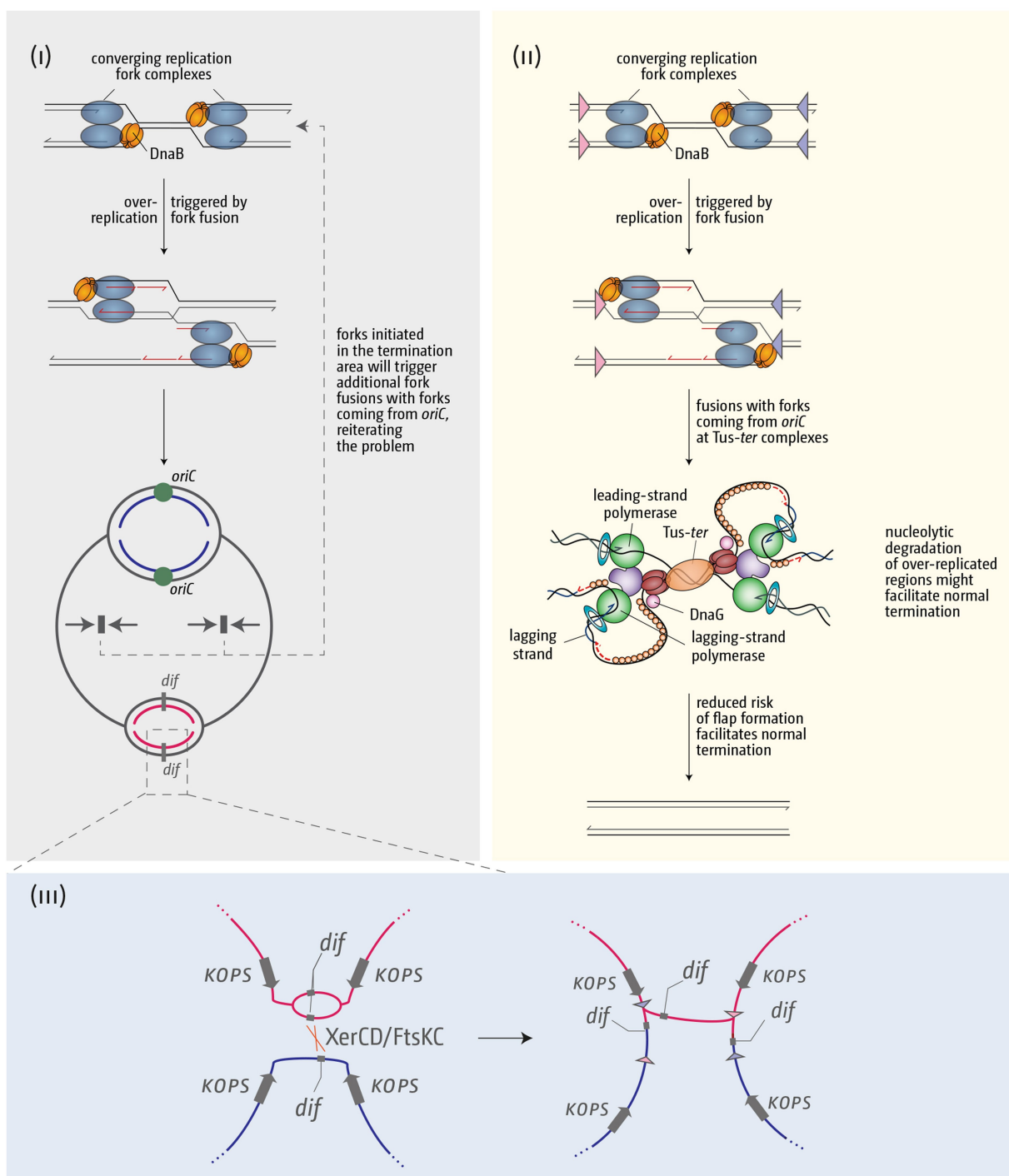
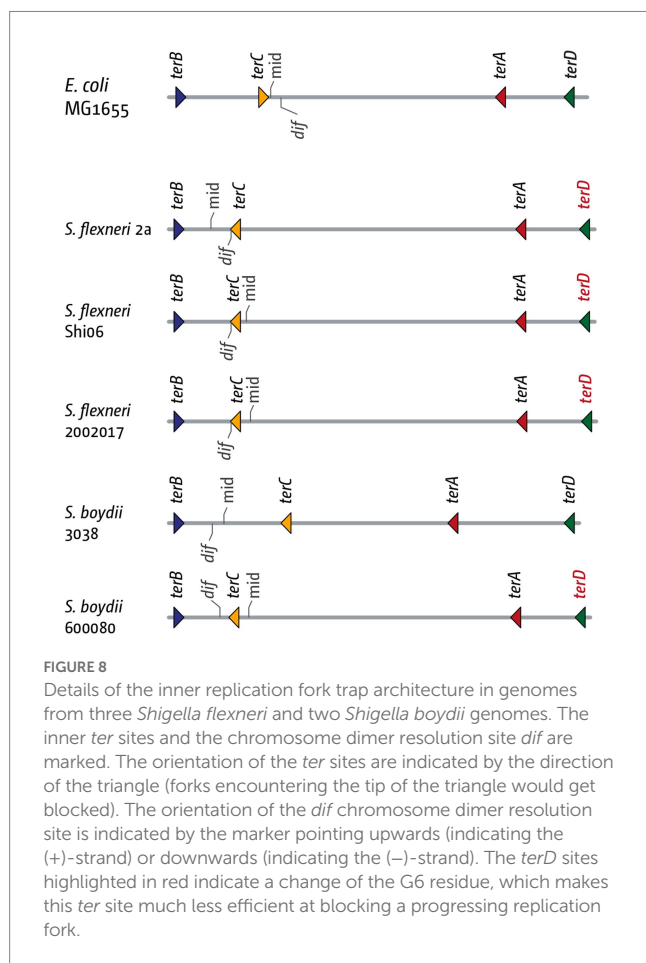


FIGURE 7
 Schematic illustrating how over-replication triggered by replication fork fusions might trigger problems for chromosome duplication and segregation, and how the presence of a fork trap area might help in resolving these issues. **(i)** Over-replication triggered in the termination area by fork fusion reactions will result in additional collisions with forks coming from *oriC*, thereby reiterating and exacerbating the problem. **(ii)** A fork fusion reaction by a freely moving fork encountering a second stably arrested at a *Tus-ter* complex is less likely to result in formation of a 3' flap and subsequent over-replication, thereby facilitating successful termination. **(iii)** If termination induces over-replication and the chromosome dimer resolution site *dif* is duplicated, this can lead to problems of the chromosome dimer resolution mechanism. See text for further details.

Similarly, if two *dif* sites are available for processing before replication is complete, which might be the case if the fork fusion point is located in a different area of the chromosome to *dif*, this will be problematic, as it could trigger unwanted processing events. Indeed, we showed recently that the doubling time of a strain in which the *dif* site is

moved approximately 100kb out of the inner termination area is significantly longer (27 vs. 22 min) (Goodall et al., 2021). The importance of limiting the number of available *dif* sites is also highlighted by the fact that in *Vibrio cholerae*, which carries two circular chromosome, the *dif* sites of the different chromosomes have



particular sequence changes, thereby avoiding inter-chromosomal attempts of processing (Castillo et al., 2017).

In this context we stumbled across an intriguing detail in *Shigella*. When analyzing the termination area in *Shigella flexneri* and *Shigella boydii* strains, we found that, in the majority of genomes investigated, the *dif* site was located between *ter* sites B and C, rather than A and C (Goodall et al., 2021). This change was accompanied with a consistent inversion of *terC*, as well as a mutation of the critical C6 base in *terD* (Figure 7). In other words, in these strains the innermost termination area is flanked by *terB* on one side, and *ter* sites C, A and D on the other, with D often inactivated, as the critical C6 is mutated (Kamada et al., 1996; Neylon et al., 2005). Thus, it seems that *dif* moves with the location of the inner termination area, or vice versa (Goodall et al., 2021). In contrast, in the absence of a replication fork trap the delay of one fork will result in progression of the opposite fork, leading to the duplication of the *dif* site before chromosome duplication is complete, generating intermediates that are not as efficiently processed at the late stages of the duplication process. Thus, by restricting the continuation of synthesis, a fork trap system is likely to alleviate all of these problems.

Conclusion

The chromosomal architecture in bacteria is quite remarkable: the strict limitation to a single replication origin (Gao and Zhang, 2008; Gao, 2015) dictates that the speed of replication directly influences

growth. Faster growth will require faster replication fork speeds, and under fast growth conditions DNA replication in *E. coli* is indeed almost 15× faster than DNA synthesis in eukaryotic cells (Pham et al., 2013). To speed up genome duplication, bacteria such as *E. coli* use overlapping rounds of replication, while the seemingly simple solution of multiple origins is not used. Both in archaea and eukaryotic cells the use of multiple origins is the norm (Early et al., 2004; Ausiannikava and Allers, 2017). What difference allows the use of multiple origins? Eukaryotic cells have much larger genomes, the duplication of which is facilitated by an increased number of replication forks (Leonard and Méchali, 2013), but this is not a satisfactory explanation, as archaeal chromosomes and also the chromosomes of, e.g., *Saccharomyces cerevisiae* are of similar size or even smaller than the *E. coli* chromosome (Belda et al., 2019) and still several origins are present.

Multiple factors will have contributed to the development of these distinct chromosomal architectures. However, as highlighted above, termination of DNA replication can trigger the formation of intermediates that cause a variety of downstream problems. These problems include chromosomal over-replication, threatening the control that restricts genome duplication to once per cell cycle, localized increases in recombination which will destabilize the genome, topological problems, which may result in secondary effects, such as increased levels of R-loops, and problems for the processes associated with the late stages of genome duplication, such as decatenation and chromosome dimer resolution. Given the large number of possible problems, it is perhaps not surprising that a large number of conserved protein activities are reported to be involved in the rapid and effective processing of fork fusion intermediates. However, it is tempting to speculate that one easy and efficient way to restrict the number of fork fusion events is by restricting the number of origins to its minimum – a single event. We believe that a fork trap system, while not essential, adds an additional mechanistic layer to the coordination of fork movement. It enables intermediates that arise in a defined area of the chromosome to be trapped, which facilitates their processing, as well as the coordination of termination with concatenation and chromosome dimer resolution (Figure 7), explaining why, once acquired, it was so consistently retained.

But why is the increased number of origins less of a problem in archaea and eukaryotic cells? Our data strongly suggest that 3' flaps are an important termination intermediate as replication fork complexes fuse in bacteria (Rudolph et al., 2010, 2013; Dimude et al., 2015; Midgley-Smith et al., 2019). 3' flap formation is driven by the replicative helicase DnaB encircling the lagging strand template (LeBowitz and McMacken, 1986). In contrast, the archaeal and eukaryotic replisomes use CMG helicase to unwind the parental duplex, which will not generate a 3' flap structure, as it is not encircling the lagging strand template (Abid Ali and Costa, 2016), which makes it likely that the precise intermediates that are generated as forks fuse differ significantly from the situation in bacteria.

However, this does not mean that the termination stage of DNA replication is unproblematic in eukaryotic cells. On the contrary, recent work by numerous labs has highlighted that the fusion of two forks needs to be carefully orchestrated as well. Similar to bacterial helicases such as RecG, the helicases Pif1 and Rrm3 play an important role in facilitating fork fusions and bringing the replication process to a successful conclusion (Steinacher et al., 2012; Deegan et al., 2019), even though replisomes appear not to slow as they move towards each other (Dewar

et al., 2015; Dewar and Walter, 2017). Furthermore, upon completion of synthesis the disassembly of the replisome components needs to be carefully coordinated (Maric et al., 2014; Moreno et al., 2014; Moreno and Gambus, 2015; Sonnevile et al., 2017; Zhou et al., 2021).

We are only beginning to understand the molecular mechanisms that are at play during this fascinating stage of the replication process. Future mechanistic studies in bacteria, archaea and eukaryotic cells will without doubt provide additional important mechanistic details of how replication is concluded efficiently and with high accuracy.

Author contributions

DG and CR wrote the initial draft of the article. DG, DW, MH, and CR were involved in extensive edits of the entire text. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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