End of the Beginning: Elongation and Termination Features of Alternative Modes of Chromosomal Replication Initiation in Bacteria

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Overview: In bacterial cells, bidirectional replication of the circular chromosome is initiated from a single origin (oriC) and terminates in an antipodal terminus region such that movement of the pair of replication forks is largely codirectional with transcription. The terminus region is flanked by discrete Ter sequences that act as polar, or direction-dependent, arrest sites for fork progression. Alternative oriC-independent modes of replication initiation are possible, one of which is constitutive stable DNA replication (cSDR) from transcription-associated RNA–DNA hybrids or R-loops. Here, I discuss the distinctive attributes of fork progression and termination associated with different modes of bacterial replication initiation. Two hypothetical models are proposed: that head-on collisions between pairs of replication forks, which are a feature of replication termination in all kingdoms of life, provoke bilateral fork reversal reactions; and that cSDR is characterized by existence of distinct subpopulations in bacterial cultures and a widespread distribution of origins in the genome, each with a small firing potential. Since R-loops are known to exist in eukaryotic cells and to inflict genome damage in G1 phase, it is possible that cSDR-like events promote aberrant replication initiation even in eukaryotes.

Introduction

Many features of chromosomal DNA replication are shared across the three kingdoms of life, including initiation from discrete origins, bidirectional fork progression, and termination by merger of opposing replication forks [1]. Whereas replication in eukaryotes is initiated from multiple origins on linear chromosomes, in bacteria most often there is a single circular chromosome whose replication is initiated from an oriC locus and proceeds bidirectionally for forks to meet in an antipodal terminus region. With this arrangement, replication and transcription of highly transcribed genes are rendered majorly codirectional in bacterial genomes. oriC-like sequences have been identified in more than 1,500 bacteria [2].

Alternative (oriC-independent) means of bacterial chromosomal replication have been characterized. These include (i) “integrative suppression” with replicons of plasmid or phage, and (ii) replication initiated from RNA–DNA hybrids or R-loops. The latter is called constitutive stable DNA replication (cSDR), whose mechanism is poorly understood. Significant perturbations, both of codirectionality between replication and transcription and of the arrangement for opposing replication forks to meet in the terminus region, are expected when bidirectional replication is not oriC-initiated.

This review explores the dynamics of fork progression and termination in Escherichia coli (gram-negative) bacterial cells exhibiting oriC-dependent and oriC-independent replication initiation to support two new concepts: (i) that when pairs of forks collide, bilateral fork reversal reactions take place; and (ii) that cSDR is characterized by stochastic replication initiation events distributed genome-wide. Table 1 summarizes relevant features and functions in E. coli that are shared in Bacillus subtilis (a gram-positive bacterium) and in eukaryotes, as is further elaborated in the text.

Replication Initiated from oriC and Its Termination

Bidirectional replication initiation at oriC is dependent on the protein DnaA, and included within the replisome complex at each fork is the 5’-3’ replicative helicase DnaB [3–6]. The forks move divergently around the circular chromosome to meet in the terminus region, and their traversed paths represent the (clockwise and counterclockwise) replicichores (Fig. 1A). Both oriC and DnaA are essential for viability.

Advancing forks may suffer disintegration [7,8], whose frequency is increased with DNA damage [7–10] or by transcription–replication conflicts [11–16]. For example, all seven ribosomal RNA operons are codirectional with the replicichores, and inversion of any of them leads to slowing or disintegration of replication forks [17–19]. Accessory helicases Rep and UvRD with 3’-5’ polarity facilitate replisome progression across DNA–protein barriers, including at sites of transcription–replication conflict [15,18–20]. Fork disintegration also occurs when one fork runs into a preceding one stalled on the same replicichore [21,22]. Reassembly of disintegrated forks is mediated by replication restart proteins acting together with the proteins for homologous recombination RecA, RecBCD, and RuvABC (Table 1) [7–10].

At the terminus region, the Tus protein binds to discrete Ter sequences and mediates polar, or direction-specific, arrest of replisome progression (by inactivating DnaB helicase) [12,23–25].

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Occurrence in the oppositely directed replisomes \[12,24,28\].

E. coli meet at, or in, the interval between most often chromosomal replication is completed when opposing forks and counterclockwise forks are terminated, and (ii) its counterclockwise end, thus enabled identification of origins and termini of replication (Fig. 1B) \[29,30\]. Analysis of copy number distributions has therefore enabled identification of origins and termini of replication [29,30].

Thus, this region contains at (i) its clockwise end, TerA where counterclockwise forks are terminated, and (ii) its counterclockwise end, TerC and TerB where clockwise forks are arrested (Fig. 1A). Hence, most often chromosomal replication is completed when opposing forks meet at, or in, the interval between TerA and TerC or TerB. However, replisome arrest at Tus-bound Ter sites is not absolute \[26,27\]. In E. coli, TerA, TerB, and TerC are present on the E. coli replicohores (Fig. 1A), oriented such as to cause arrest only of the oppositely directed replisomes \[12,24,28\].

Three essential replicative helicase in replisome (DnaB homohexamer, 5'-3' polarity) \[4,112\].

Facilitation of replisome progression by accessory helicases (PcrA) \[15,116–118\].

Fork disintegration and replication restart Yes [119–121] Yes [1,9,10,96,122].

DNA repair by homologous recombination Yes [121] Yes [10,121–126].

a. Recombinase (RecA) Similar to that in E. coli [121] Yes (Rad51) \[10,121–125\].

b. Exonuclease resection at double strand ends (RecBCD) Yes (AddAB) \[122,127–129\].

c. Enzymes for Holliday junction migration and resolution (RuvABC) Yes (RuvAB, RecU, RusA) \[121,132\].

Replication fork reversal at stalled replisomes Postulated, including during phage (SPP1) replication \[15,133,134\] Yes [10].

Completion of replication termination by merger of opposing replication forks Similar to that in E. coli \[12,24,25\].

Replication–transcription codirectionality in highly transcribed genes (such as rRNA genes) Similar to that in E. coli \[15,120,137,138\].

Rho-dependent termination of nascent untranslated (including antisense) transcripts Yes [140] No.

Transcription-associated R-loops Not demonstrated Yes [80–94,141,142].

a. R-loop prevention by topoisomerase I action Not demonstrated Yes [85,143].

b. RNase H Yes [144,145].

c. RecG helicase Similar to that in E. coli \[132,147,148\] (but see footnote a below) Not demonstrated.

cSDR Not demonstrated Not demonstrated.

In an assay involving copy number determination of an R-loop dependent plasmid in E. coli, RecG from another gram-positive bacterium Streptococcus pneumoniae was shown to be active as an R-loop helicase but, unexpectedly, RecG from B. subtilis was inactive \[147\].

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Thus, this region contains at (i) its clockwise end, TerA where counterclockwise forks are terminated, and (ii) its counterclockwise end, TerC and TerB where clockwise forks are arrested (Fig. 1A). Hence, most often chromosomal replication is completed when opposing forks meet at, or in, the interval between TerA and TerC or TerB. However, replisome arrest at Tus-bound Ter sites is not absolute [26,27]. In addition to TerA, -B and -C, seven other Ter sequences are present on the E. coli replicohores (Fig. 1A), oriented such as to cause arrest only of the oppositely directed replisomes [12,24,28].

Copy Number Analysis in Chromosome Replication Studies

When replication forks advance from origin to terminus in cells of an asynchronously dividing cell population, a decreasing gradient of gene copy numbers is expected from the former to the latter (Fig. 1B) [29,30]. Analysis of copy number distributions has therefore enabled identification of origins and termini of replication [26,27,31–36] as well as of chromosome rearrangements [37]. Two caveats are (i) that copy numbers can change on account not only of fork progression but also of recombination (for example, tandem amplification [38]) or DNA degradation [39,40]; and (ii) that the values represent an average of all cells in a population, which may comprise distinct subpopulations including inviable cells [38,41].

When Replisomes Collide: Evidence for Bilateral Replication Fork Reversals

Replication fork reversal is the process by which nascent leading and lagging daughter strands at a fork are extruded to anneal to one another, thus forming a cruciform or “chicken-foot” structure. Such extrusion could occur when replisome progression is halted for any reason, and would be promoted by accumulation of positive supercoils ahead of the fork. Fork reversals can competitively be either limited by “end-resection” activity of the RecBCD complex, or exacerbated by the RuvABC proteins that catalyze branch migration and cleavage at Holliday junctions [19,42–43], reviewed in [10,46]. In RecBC-deficient strains, fork reversal is also accompanied by excessive chromosome degradation (which may indeed seem paradoxical given that RecBCD is itself a potent DNA exonuclease), that is mediated by RecJ nuclease [19].
Fig. 1. Features of oriC-initiated replication in E. coli. (A) Depiction of oriC, TerA, TerB and TerC loci on the 100 minute long circular E. coli chromosome, and of the clockwise and counterclockwise replichores; locations of the seven other Ter sites are also shown. (B) Schematic depiction of the copy number gradient, from oriC to Ter, created by the different extents to which replication forks have progressed on a single replichore in individual cells of an asynchronously dividing population. Aggregate copy numbers at the indicated positions are given at the bottom, but these are only illustrative and not to scale.

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Kuong et al. [40] and Rudolph et al. [41] have shown that chromosomal terminus region copy numbers are severely reduced in RecBC-deficient strains (the former studies were done with thymine starvation). This suggests that irreparable chromosome breakage and degradation occurs in the terminus region of some proportion of recBC mutant cells which, for example, may result from bilateral fork reversals provoked by head-on collisions between two replisomes, as depicted in Fig 2. Additional experiments are required to confirm this hypothesis. Since collisions between pairs of replisomes are a common feature of replication termination in all organisms [25,47–49], it is also possible that consequential fork reversals may be universal.

Chromosome Replication in Absence of oriC or DnaA: Integrative Suppression

In integrative suppression, the replication origin of a plasmid or phage is integrated into the chromosome of a strain defective in oriC or DnaA [26,27,33–36,50–53]. In general, a strain's health is more compromised when the exogenous origin has integrated further from oriC, and when replication is unidirectional rather than bidirectional. Retrograde replication fork progression (towards oriC) in strains with ectopic origins is slow [27,54], presumably because codirectionality between replication and transcription is lost, providing support to the model of impedance of fork movement by head-on transcription [17–19].

For strains where the exogenous origin is integrated at oriC-distal sites, the terminus region is replicated (as expected) by the fork which traverses the shorter arc between it and the integration site, but additionally there is a sharp decrease in copy numbers immediately before the Ter site that arrests its passage [26,27]. A similar decrease in copy numbers proximal to the Ter arrest site of a prematurely arriving fork is evident in a strain possessing two chromosomal replication origins [41]. It is possible that these decreases are related to changes in fork architecture at the arrest sites, leading to DNA degradation by endo- and exonucleolytic enzymes.

Chromosome Replication in Absence of oriC or DnaA: cSDR

Another means to confer viability to cells lacking oriC or DnaA is cSDR, wherein transcription-associated R-loops serve as primers for initial DNA synthesis following which replication forks are established by the mechanisms of replication restart [55,56]. Enzymes RNase HI (rnhA-encoded) and RecG (recG-encoded) act to eliminate R-loops by hydrolysis and by unwinding, respectively, and DNA synthesis by cSDR has been demonstrated in both rnhA and recG single mutants (while the double-deficiency is lethal) [55]. R-loops similarly initiate replication in ColE1 plasmids [57,58]. cSDR has also been implicated in stress-induced mutagenesis and genome instability [59].

cSDR Origin Sites in RNase HI-Deficient Mutants

By examining the copy number gradient in rnhA mutants lacking oriC-initiated replication, the late Kogoma’s group reported several putative replication initiation sites (termed oriKs), at least two of which were in the chromosomal terminus region [55]. Madiuke et al. [60] have revisited this question through a deep sequencing approach, and their results have again demonstrated a prominent copy number peak in the terminus region of rnhA mutants. However, this peak was abolished in a Tus-deficient derivative, leading the authors to suggest that it may not represent an oriK site but instead could be a consequence of trapping by polar Ter sites of replication forks that were initiated outside, and had then progressed into, the terminus region [60]. This idea is further developed in my model proposed below. Furthermore, no oriK locus was detected in a chromosome-wide search for sequences that could confer autonomous replication ability in an RNase HI-deficient strain [38,61]. Thus, a definitive identification of the so-called oriK sites for cSDR has remained elusive.

Where Do R-loops Occur in the E. coli Genome?

One way to identify replication initiation sites in cSDR would be to determine the locations of R-loops in the genome, even while it is recognized that their occurrence is necessary but may not be sufficient for establishing origin activity [62]. R-loop mapping studies have not been reported for rnhA or recG mutants, but they have been done [63] for a mutant defective in Rho-dependent transcription termination (RDTT) as explained below.

RDTT is a process by which nascent non-rRNA transcripts that are not being simultaneously translated are prematurely terminated. RDTT-deficient mutants exhibit an increased prevalence of R-loops [63–65], which is assumed to arise from the reassnealing of nascent untranslated transcripts with upstream DNA [66,67]. R-loop formation in these situations is facilitated also by backtracking of RNA polymerase leading to stalled or arrested transcription elongation complexes [68,69], but why this is so is unclear.

Fig. 2. Model of bilateral fork reversal reaction at a site where oncoming replisomes meet during replication termination. doi:10.1371/journal.pgen.1004909.g002
In an RDTT-deficient mutant, R-loops are distributed genomewide, being generated from both sense and antisense transcripts [63]; Peters et al. [70] have also shown that antisense transcription is increased when RDTT is compromised (reviewed in [71]). Therefore, it is likely that oriK sites for cSDR are also widespread, and that they may indeed be stochastically different amongst individual cells in a population. This would explain the earlier findings [60,61] that no distinct oriK sites were unambiguously identified in RNase HI-deficient mutants.

**cSDR in RecG-Deficient Mutants**

**cSDR with RNase HI- or RecG-deficiency: Similar findings, different models**

Copy number studies in both rnhA [60] and recG [41] mutants have demonstrated the similar occurrence in them of Tus-dependent (i) peak in the chromosomal terminus region, and (ii) inversion of the classical oriC-peaked curve when replication initiation from oriC is abolished. However, cSDR in the recG mutant has been explained to be the consequence of aberrant replication reinitiation events following fork collisions [41,72].

According to this model [41], when opposing forks meet in the terminus region, DnaB helicase acts to unwind and extrude the oncoming fork’s leading strand at its 3’ end, which then serves as a substrate for aberrant replication restart unless RecG and at least one of three single-strand DNA 3’ exo¬nuclease is lethal [73], which has been attributed to excessive occurrence of such over-replication in these cells. However, the source of origin of forks which are postulated to collide in the terminus region to mediate cSDR in recG mutants has not been explained, since these strains were also DnaA-deficient [41].

This raises the question of replication initiation in cSDR occurring by completely different mechanisms in rnhA and recG mutants, the former from R-loops [55,60] and the latter from fork collisions in the terminus region [41]. However, the similarities cited above would suggest that cSDR in both indeed operates by a common mechanism, as is further explored below. An additional similarity is that, just as with RecG deficiency, RNase HI deficiency is also lethal in the combined absence of the three 3’ exo¬nuclease [73].

**A model invoking subpopulations with distinct replication dynamics during cSDR**

The sharpness of the observed copy number peak in the terminus region of recG mutants is inconsistent with the excessive reinitiation model [41], which would predict that copy numbers exhibit a plateau (with no peaks) across this entire interval between TerA and TerB or TerC (since replication reinitiation anywhere within this region will duplicate all markers between the Tus-bound Ter boundaries). An alternative way to explain the observed peak in the mid-terminus region of a recG or rnhA mutant is to assume that its copy number curve is a composite of distributions from subpopulations with one or more of three different kinds of replication initiation events, as represented in Fig. 3.

For the purpose of this depiction, 60% of replication initiations are envisaged to have occurred from oriC (Fig. 3A), and 20% each from R-loops in the counterclockwise and clockwise replichore arms (Figs. 3B and 3C, respectively). Nevertheless, a single cell may harbor more than one category of replication event: for example, a simple representation of the percentages above would have it that for every three cells in the culture per generation, one suffers a (supernumerary) cSDR initiation event on the clockwise replichore, another a similar event on the counterclockwise replichore, whereas all exhibit oriC-initiated replication.

For cSDR events initiated from sites on the counterclockwise replichore (Fig. 3B), retrograde progression of forks towards oriC would be slow (as noted in other cases earlier [27,54]), whereas they would progress smoothly towards and beyond Ter/C into the terminus region to be arrested at TerA; the small proportion of forks that overcome arrest would then progress in retrograde direction beyond TerA. Since R-loops are evenly distributed [63], cSDR origins are also likely to occur at a uniform, but low, probability across the genome, such that the copy number for an arbitrary locus on the counterclockwise arm is higher the further its distance from oriC (which is opposite to that with DnaA-mediated initiations from oriC; compare Figs. 3A and 3B).

The earlier studies [26,27,41] have also indicated that prolonged arrest of replication forks at a Ter site, in the absence of arrival of forks of the opposite replichore, is associated with a sharp copy number drop in the region preceding the fork arrest site (which is depicted in Figure 3B adjacent to TerA, in the interval between TerA and TerC or TerB). The mirror symmetrically reverse situation would apply for cSDR initiation events on the clockwise replichore, as shown in Figure 3C.

The composite pattern for the entire population, derived by summation of the three distributions above, is shown in Figure 3D. Two features of the data reported for mutants lacking RecG [41] or RNase HI [60] are recapitulated here, namely, a peak in the mid-terminus region and a smaller enrichment of oriC-proximal to oriC-distal markers (compare Figs. 3A and 3D). As has also been suggested earlier [41], many cSDR events may likely contribute only to copy number values without concomitantly increasing viable cell numbers, since every event would not necessarily lead to duplication of the entire chromosome.

With the same assumptions, the copy number distribution in an rnhA or recG mutant that is additionally defective for DnaA can be derived as the approximate composite of Figs. 3B and 3C, as depicted in Fig. 3E. The derived curve broadly recapitulates the inversion in these mutants of the “classical” curve so that the peak and trough are now at the terminus and oriC, respectively [41,60].

In strains exhibiting cSDR, the frequency of replication fork disintegration events is expected to be elevated when replication advance towards oriC; this would explain their dependence for viability on proteins of replication restart and homologous recombination [55,56]. Since R-loop prevalence is less upon loss of RecG than of RNase HI [55], cSDR-mediated viability of a recG dnaA mutant requires the presence of additional mutations in Tus and RNA polymerase (rpoB35) [41]. While absence of Tus permits passage of counterdirectional forks across Ter sites, rpoB35 mitigates the adversity associated with transcription—replication conflicts [44,68,74]; in cSDR, rpoB35 would promote retrograde fork progression both from cSDR initiation sites and in regions beyond the Ter sites.

**Comparisons in Other Organisms**

The similarities listed in Table 1 between *E. coli* and *B. subtilis* would suggest that the models proposed here for the former may apply to the latter, although cSDR has so far not been demonstrated in *B. subtilis*. Archaeal and eukaryotic DNA replication mechanisms are very similar [1,75–77], and in the archaeon *Haloferax volcanii*, the circular chromosome possesses four replication origins, but derivatives in which all were deleted unexpectedly exhibited greater fitness than the parental strains [78]; a cSDR-like mechanism has been proposed in the originless...
Fig. 3. Predicted copy number distribution patterns for different categories of replication events in recG or rnhA mutants. For all curves, positions of oriC, TerA, and TerC or TerB (TerC/B), are marked by the interrupted vertical lines; and copy number values are plotted on a linear instead of log scale to enable comparison with curves shown in Rudolph et al. [41]. (A–C) Three categories of replication events are shown, comprising those with forks initiated, respectively, (i) at oriC, DnaA-mediated (60%); (ii) on the counterclockwise replichore at various locations, R-loop mediated (20%); and (iii) on the clockwise replichore at various locations, R-loop mediated (20%). An individual cell in the population may harbor more than one category of event (see text). On the right in each of the three panels is a schematic depiction of progression of forks, each beginning at a solid circle and progressing to the position of arrowhead; in panels B and C, terminus region chromosomal DNA degradation (proximal to the sites of fork arrest at Ter) is shown as interrupted lines on the arcs, but retrograde fork advancements towards oriC (which are expected to occur at
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