Robust linear DNA degradation supports replication–initiation-defective mutants in *Escherichia coli*

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Abstract

RecBCD helicase/nuclease supports replication fork progress via recombinational repair or linear DNA degradation, explaining recBC mutant synthetic lethality with replication elongation defects. Since replication initiation defects leave chromosomes without replication forks, these should be insensitive to the recBCD status. Surprisingly, we found that both *Escherichia coli dnaA46*(Ts) and *dnaC2*(Ts) initiation mutants at semi-permissive temperatures are also recBC-colethal. Interestingly, *dnaA46* recBC lethality suppressors suggest underinitiation as the problem, while *dnaC2* recBC suppressors signal overinitiation. Using genetic and physical approaches, we studied the *dnaA46* recBC synthetic lethality, for the possibility that RecBCD participates in replication initiation. Overproduced DnaA46 mutant protein interferes with growth of *dnaA46* cells, while the residual viability of the *dnaA46* recBC mutant depends on the auxiliary replicative helicase Rep, suggesting replication fork inhibition by the DnaA46 mutant protein. The *dnaA46* mutant depends on linear DNA degradation by RecBCD, rather than on recombinational repair. At the same time, the *dnaA46* defect also interacts with Holliday junction-moving defects, suggesting reversal of inhibited forks. However, in contrast to all known recBC-colethals, which fragment their chromosomes, the *dnaA46* recBC mutant develops no chromosome fragmentation, indicating that its inhibited replication forks are stable. Physical measurements confirm replication inhibition in the *dnaA46* mutant shifted to semi-permissive temperatures, both at the level of elongation and initiation, while RecBCD gradually restores elongation and then initiation. We propose that RecBCD-catalyzed resetting of inhibited replication forks allows replication to displace the “sticky” DnaA46(Ts) protein from the chromosomal DNA, mustering enough DnaA for new initiations.

Keywords: synthetic lethality; oriC; DnaA; DnaG; DnaN; RecA; RecBCD; RuvA; RecG; Rep; recombinational repair; linear DNA degradation; chromosomal DNA synthesis; replication initiation; replication elongation

Introduction

Chromosomal replication in *Escherichia coli* begins at oriC, when, as detected in vitro, the DnaA protein in complex with ATP binds to specific short sequences in the origin DNA called DnaA boxes (Fuller and Kornberg 1983; Fuller et al. 1984; Sekimizu et al. 1987). The oriC DNA has several high- and low-affinity DnaA boxes, promoting limited DnaA polymerization into a spiral multisubunit DnaA-ATP filament (Fig. 1a), with the oriC DNA wrapped along the outside of the filament (Fuller et al. 1984; Erzberger et al. 2006; Miller et al. 2009). The resulting positive supercoiling in the oriC DNA is proposed to cause compensatory hyper-negative supercoiling within the nearby AT-rich DNA sequence, promoting its unwinding into a single-strand bubble (Erzberger et al. 2006) (Fig. 1a). In the next step, as observed in vitro, DnaC loads 2 DnaB helicase hexamers at the opposite corners of the single-strand bubble (Funnell et al. 1987; Marszalek and Kaguni 1994), followed by recruitment of DnaG primase and, after the synthesized primer is loaded with the DnaN clamp, of the replisome itself, that begins bidirectional DNA synthesis (Fang et al. 1999) (Fig. 1a). Mapping of the priming sites around oriC shows, however, that the initial replication is unidirectional, going away from the DnaA-bound cluster, while the other direction is activated later and away from oriC, both in vivo (Kohara et al. 1985) and in vitro (Fang et al. 1999). Most likely, the second fork is activated, as the first Okazaki fragment on the lagging strand of the “first fork” becomes its leading strand. The progress of the fork in the opposite direction displaces the DnaA filament off oriC DNA (Fig. 1a), using the DnaN-riding Hda protein (as explained later).

Apart from oriC, high-affinity DnaA boxes are also found at the dnaA locus, that converts initiation-competent ATP-DnaA into initiation-inactive ADP-DnaA and thus regulates initiation by controlling the levels of ATP-DnaA (Kitagawa et al. 1998; Kasho and Katayama 2013). DnaA is also known to regulate transcription of several genes, including itself (Atlung et al. 1985; Braun et al. 1985; Kücherer et al. 1986) and, generally, binds to more than 300 sites all over the chromosome (Hansen and Atlung 2018).

Chromosomal replication in bacteria is extremely fast and accurate under optimal conditions, but since only 2 replication forks (RFs) traverse the entire chromosome per cell cycle, it takes *E. coli* a minimum of 40 min to finish chromosome duplication.
Helmstetter 1987). Part of the reason replication cannot be faster is that replisomes are slowed down by encounters with DNA damage, heavily transcribed genes or tightly bound proteins, which cause RF stalling and even disintegration (Bierne and Michel 1994; Kuzminov 1995a, 1995b; Cox et al. 2000) (Fig. 1b). Disintegrated RFs are reassembled by recombinational repair enzymes RecBCD, RecA and RuvABC/RecG (Kuzminov 1999); DNA synthesis at a restored or abandoned fork structure resumes after replisome reloading by PriA (Michel and Sandler 2017). For example, the rep mutant in E. coli, famous for its slow RFs (Lane and Denhardt 1975), is susceptible to fork stalling and disintegration (Michel et al. 1997). This makes the rep mutant synthetic lethal with the recBC defect (Uzest et al. 1995), meaning that the RecBCD function is essential for the rep mutant survival. In general,
synthetic lethality (or “colethality”—a simpler term which we use sometime) is inviability of a combination of 2 viable mutants (Guarente 1993; Nijman 2011).

The RecBCD enzyme is a helicase-nuclease that vigorously degrades linear DNA, but having recognized a Chi-site, generates 3' overhangs at double strand DNA ends (Dillingham and Kowalczykowski 2008), which are then used by the RecA protein for homology-guided strand invasion into an intact duplex DNA (Cox 1993; Kuzminov 1999) (Fig. 1b, the left pathway). The invasion creates a Holliday junction (HJ) intermediate, which is resolved by either the RuvABC resolvase or by the RecG duplex DNA pump (Fig. 1b, the left pathway) (Kuzminov 1999). In the absence of RecBCD, the stalled forks in the rep mutants undergo RuvAB-dependent RF reversal forming an HJ, which is eventually cleaved by RuvC (Seigneur et al. 1998) (Fig. 1b, the right column). A similar stalling, reversal and cleavage of the fork in the other replisome causes release of linear sister chromosome arm (detectable as chromosome fragmentation in pulsed-field gels) and the rep recBC synthetic lethality (Uzest et al. 1995; Michel et al. 1997). If RecBCD is present, however, it degrades the extruded DNA from the reversed fork, preventing such double strand breaks by RuvC and allowing the fork to reset (Seigneur et al. 1998) (Fig. 1b, the bottom right).

Apart from rep, various E. coli replication elongation mutants, that experience fork problems, either in the replicative helicase DnaB, or in the β-clamp, α, or ψ subunits of the replicative polymerase, have been shown to experience RuvABC-dependent double strand breaks as a result of fork reversal and to require RecBCD for viability (Michel et al. 1997; Flores et al. 2001; Grompone et al. 2002). At the same time, mutants impaired for replication initiation functions, dnaA and dnaC (Fig. 1a), were assumed to be indifferent toward recombinational repair status of the cell, as without initiation there are no RFs in the chromosome that may require repair. True, DnaC also functions away from the cell, as without initiation there are no RFs in the chromosome and DNA in duplicate agarose plugs. The cells in plugs were lysed for micrograph, after 18–24 h of incubation of plates at room temperature, aliquots were removed, serially diluted, and spotted on LB plates. Colonies were counted while still small under stereo-microscope, after 18–24 h of incubation of plates at room temperature.

**Materials and methods**

**Bacterial strains, growth conditions, and chemical reagents**

Escherichia coli strains used in this study are all K-12 derived and are described in Supplementary Table 1. The strains were grown in LB (per 11: 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, pH to 7.4 with 250 µl of 4 M NaOH; LB agar contained 15 g of agar/l of LB broth) at 28°C unless mentioned otherwise.

**Plasmids and cloning**

Plasmids are described in Supplementary Table 2. Plasmids pNKR416 and pMob-dnaKJ were provided by Benedicte Michel (CNRS, France). For the construction of pPR1 (dnaA + dnaN+) plasmid, genomic DNA of MG1655 and pMTL20 were both digested with NcoI and ligated to create a plasmid library. The plasmid library was transformed into DH5α dnaA46 strain and plated at 42°C to select for suppressors of temperature sensitivity of DnaA46. One clone containing insert was selected and called pPR1. pPR1 was digested with XhoI and religated to create the dnaA+ (dnaN−) plasmid pPR7. pPR1 was digested with NcoI, religated and transformed into DH5α dnaA46 to create a plasmid with insert in the opposite orientation. Plasmid pPR2 was isolated from suppressors that grew and checked for inversion of orientation. pPR2 was digested with Sphi and religated to create the dnaN+ (dnaA−) plasmid pPR8.

**Cloning dnaA and dnaA46 genes in pMTL20**

Primers GCTATCCATGGTACGGGCTGATG and GCTATCAAGCTTGGCACCATTTCACATCGC were used to amplify the dnaA+ and dnaA46 genes. Upon amplification the insert and vector were digested with NcoI and vector were digested with NcoI and HindIII, ligated and transformed into DH5α dnaA46 to select for suppressors. Plasmid DNA was isolated from the suppressors, and inserts were verified by sequencing.

**Cloning of hslUV+ into pMTL20**

Primers CTCTCCGGAATTCCGACTTGTGGTTGAAGTCCG and CTGCCATCGGTAGAAAAATGATTGAACGCG were used to amplify the wild type hslUV+ genes. The amplicon and the plasmid pMTL20 were digested with EcoRI and NcoI and ligated.

**Spot test**

Overnight cultures were diluted 100-fold to subculture and then grown at 28°C to OD600 = 0.1–0.15. Strains containing plasmids were subcultured in the presence of antibiotic to maintain the plasmid. Serial 10-fold dilutions were made in 1% NaCl, and 10 µl of the original culture, as well as all dilutions up to 10−5 were spotted on LB plates and incubated at the indicated temperatures for 24–48 h (to yield colonies of similar sizes).

**Viability assay**

Overnight cultures were diluted 100-fold and grown at 28°C to OD600 = 0.1–0.15. The cultures were shifted to 39°C either directly or were further diluted 30-fold, shaken at 28°C for 20 min to acclimate to the diluted conditions and moved to 39°C. At appropriate times, aliquots were removed, serially diluted, and spotted on LB plates. Colonies were counted while still small under stereo-microscope.

**Origin and terminus kinetics**

Overnight cultures were subcultured 100-fold and grown at 28°C to OD600 = 0.1–0.15, when the cultures were further diluted 30-fold, shaken at 28°C for 20 min to acclimate to the diluted conditions and moved to 39°C. Twenty milliliters of recBC dnaA culture, 10 ml of dnaA, recBC dnaA seqA::pRL27 and recBC dnaA hslUV::pRL27, and 10, 8, 3, 3, and 3 ml of recBC cultures were taken at, correspondingly, 0, 1, 2, 3, and 4 h after shift to 39°C to isolate DNA in duplicate agarose plugs. The cells in plugs were lysed for 16 h at 65°C in the lysis buffer (see the next section for its
composition). After lysis, the plugs were washed with 1 ml of TE for 10 min thrice, in 1 ml of 0.25 N HCl for 20 min, in 1 ml 0.5 M NaOH for 20 min and in 1 M Tris–HCl for 20 min. The DNA from plugs was transferred using vacuum transfer and hybridized with either the probe for the origin or the terminus, which were described before (Kouzminova and Kuzminov 2012), for 24 h in hybridization buffer (5% SDS, 0.5 Na2HPO4, pH 7.4), then washed with 0.1× hybridization buffer and exposed to a phosphorimager screen.

**Quantification of chromosome fragmentation**

Overnight cultures were subcultured 100-fold and grown at 28°C to OD600 = 0.1–0.15 in the presence of 3P label, as described (Khan and Kuzminov 2012), then half of the cultures was moved to either 37°C or 39°C. Depending on the culture density, 1–25 ml aliquots were removed after 4 h of shaking, and cells were collected by centrifugation. Cell pellets were resuspended in 60 µl TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), mixed with 5 µl of 5 mg/ml Proteinase K (Roche Applied Science, final concentration in plugs is 200 µg/ml) and 65 µl of molten agarose in lysis buffer (1.2% agarose in 1% laurylsarcosine, 50 mM Tris–HCl, 25 mM EDTA, pH 8.0) and poured into plug molds (Bio-Rad). The plugs were lysed for 16 h at 65°C in the lysis buffer. Half-plugs were inserted into the wells of 1% agarose in 0.5 Tris–borate–EDTA buffer. The gel was run at 12°C in Bio-Rad CHEF-DR II pulsed field gel electrophoresis system, at 6 V/cm, with initial switch time 60 s and the final switch time 120 s, for 20 h. The gel was then dried and exposed to a phosphorimager screen. The exposed screen was analyzed with the FLA-3000 fluorescent image analyzer (Fujifilm). The data were analyzed using Image Gauge version 3.41 software (Fujifilm). The percentage of chromosomal fragmentation was calculated as signal in the lane below the well divided by the total signal in the lane + well and multiplied by 100.

**Rate of DNA synthesis**

Overnight cultures were subcultured 100-fold and grown at 28°C to OD600 = 0.1–0.15, 200 µl aliquots were taken at each time point and mixed with 1 µCi of 3H-dt, and the reaction was incubated at 39°C for 2 min, after which 5 ml of ice-cold 5% TCA was added to stop further incorporation. The TCA precipitate was collected on filters (Whatman, grade GF/C) and washed with 5 ml of 5% TCA, followed by 5 ml of ethanol. One hundred microliters of 100 mM KOH was spotted on the filter and dried, to quench fluorescence. The filters were incubated with scintillation fluid overnight before counting. 3H counts of the filters were measured in Beckman-Coulter LS 6500 scintillation counter.

**Detection of branched DNA structures**

Overnight cultures were subcultured 100-fold and grown at 28°C to OD600 = 0.1–0.15, when the cultures were diluted 30-fold, shaken at 28°C for 20 min to acclimate to the dilution and moved to 39°C for 4 h. DNA was isolated from 25 ml of dnaA, 50 ml of dnaA recBC, and from 1 ml of recBC strain by phenol–chloroform extraction. Briefly, cell pellet was suspended in 50 µl of 30% sucrose in TE and lysed with 350 µl of 2% SDS in TE after mixing by inversion and 5 min at 65°C. The lysate was extracted first with 400 µl of phenol, then with 200 µl of phenol/200 µl of chloroform mixture and, lastly, with 400 µl of chloroform. The aqueous phase was transferred to a fresh tube, and, after adding 20 µl of 5 M NaCl and 1 ml of ethanol, DNA was precipitated by multiple tube inversions. The pellet was dissolved in 500 µl TE, and DNA was reprecipitated using NaCl and ethanol. Four hundred nanograms of DNA for each strain was digested with XmnI or Stul + Ndel, and the reaction was run in 0.7% agarose on 1× Tris-acetate–EDTA buffer. The gel was treated with 0.25 N HCl for 20 min, 0.5 M NaOH for 20 min, and 1 M Tris–HCl pH = 8.0, for 20 min, DNA was transferred to nylon membrane by capillary transfer, and the membrane was hybridized with oriC-specific probe.

**Results**

**The phenomenon of RecBCD-dependent replication initiation**

Coletality of dnaA recBC

As mentioned in the Introduction, synthetic lethality is regularly observed when recombinational repair defects are combined with replication elongation defects (Michel et al. 1997, Flores et al. 2001; Grompone et al. 2002). Here, we unexpectedly found coletality between defects in recombinational repair and replication initiation. The dnaA46 allele is progressively defective in binding ATP at higher temperatures (Carr and Kaguni 1996), so it no longer supports cell growth above 40°C due to a block in replication initiation (Hirot a et al. 1970), but it still forms (smaller) colonies at 39°C, its semipermissive temperature (Fig. 1c). The recB270 recC271 (recBC(Ts)) mutant is disabled for both DNA degradation and repair at 37°C and above (Kushner 1974; Kouzminova and Kuzminov 2004), but still spots essentially like WT at 39°C (Fig. 1c). At the same time, the dnaA46 recBC(Ts) double mutant struggles to grow at 39°C (all strains show normal growth at 28°C (Fig. 1c)—making 39°C nonpermissive for the double mutant. Another temperature-sensitive allele, dnaA601, that carries the same mutation as dnaA46 in the ATP-binding site (Hansen et al. 1992), shows a similar coletality with recBC(Ts) (Fig. 1c). Unless otherwise mentioned, we have used the dnaA46 and recBC(Ts) alleles for the rest of our study; henceforth, we will refer to these 2 mutations simply as dnaA or recBC.

To see whether the inability of the dnaA recBC mutant to form colonies at the nonpermissive temperature reflects stasis or actual death, we shifted the 4 strains to 39°C while still in the liquid cultures and continued incubation for 8 more hours. As expected, the WT and recBC mutant strains grew to saturation, while the titer of the dnaA mutant increased slowly (Fig. 1d), consistent with lighter colonies in the plating assay (Fig. 1c). The titer of the dnaA recBC mutant was mostly static (Fig. 1d), suggesting that 39°C was inhibitory for the double mutant, rather than lethal. However, diluting the cultures 30 times or deeper before shifting them to 39°C, while still supporting viability of the single dnaA mutant, does lead to fast and dramatic loss of titer in the double mutant cultures (Fig. 1e). This low-cell-density-dependent coletality of the dnaA recBC double mutant explains why we always observed residual growth in the highest density spot of the strain (Fig. 1c). Our subsequent physical analyses have 2 conditions for dnaA recBC mutant: the undiluted static one (Fig. 1d) vs the diluted lethal one (Fig. 1e). Physical assays with diluted cultures, although of higher distinguishing power, were less practical because they required significant culture volumes.

**Other synthetic lethal combinations**

The dnaN gene, which encodes the DNA clamp that makes the DNA Pol III processive (Fig. 1a), is immediately downstream of dnaN in the same operon (Sakakibara et al. 1981). The dnaN159(Ts) defect (henceforth dnaN) leads to RF stalling at nonpermissive temperatures and, as many other elongation defects, is coletal in combination with the recBC defect at its semipermissive temperature of 37°C (Fig. 1f) (Grompone et al. 2002). Since DnaA autoregulates itself transcriptionally by binding to the DnaA box
at the dnaA promoter (Atlung et al. 1985; Braun et al. 1985; Kücherer et al. 1986), the observed dnaA recBC co莱thality could have been due to dnaN expression problems instead. We tested this possibility by complementing the dnaA recBC strain with the functional dnaA or dnaN genes. We found that, while dnaA-only expressing plasmid could rescue the dnaA recBC co莱thality, the dnaN-only expressing plasmid failed to do so (Fig. 1g). At the same time, we confirmed that the dnaN recBC co莱thality is due to a defective dnaN gene, as it was complemented by the dnaN-expressing plasmid, but not by the dnaA-expressing plasmid (Fig. 1g). Thus, the dnaA recBC co莱thality is due to the dnaA defect, rather than a cryptic dnaN defect.

Besides its main DNA clamp function, DnaN also plays a role in modulating initiation at oriC, as the DnaN–Hda protein complex stimulates ATP–DnaA disassembly from the origin DNA via ATP hydrolysis (Fig. 1a) (Kato and Katayama 2001). Thus, the dnaN recBC strain could be lethal due to a defect in initiation regulation (we will test this possibility later). DnaC is the DnaB inhibitor-chaperone and the second E. coli function specific for replication initiation (Fig. 1a) (Wickner and Hurwitz 1975; Wahle et al. 1989). Two types of dnaC(Ts) mutants are known, all completely defective at 42°C: dnaC1 and dnaC2 block replication elongation (Carl 1970; Wechsler 1975), while dnaC2 and dnaC28 block replication initiation (Carl 1970, Schubach et al. 1973). To explore the possibility that inactivation of any initiation function makes recBCD mutants inviable, we tested the viability of the dnaC2(Ts) recBCD mutant. Unexpectedly, we found that the dnaC2(Ts) defect (henceforth dnaC) is also synthetic lethal with the recBCD defect at the semipermissive temperature of 37°C (Fig. 1h). Thus, various problems with chromosomal replication initiation lead to RecBCD-dependence.

Suppressors of the dnaA recBC co莱thality

To understand the nature of co莱thality in the dnaA recBC, dnaN recBC, and dnaC recBC double mutants, we isolated suppressors for each of these combinations after insertional mutagenesis. Suppressors were isolated as colonies growing at the nonpermissive temperature for the given co莱thal (39°C for dnaA recBC, 37°C for either dnaN recBC or dnaC recBC) and, before identification, were confirmed to still suppress after reintroduction into the original co莱thal mutant. If the 3 co莱thalities had a common nature, they would have been suppressed by inactivation of the same or similar functions.

The dnaA recBC co莱thality was suppressed by insertions at only 2 loci (Fig. 2a; Supplementary Fig. 1, a and b). We found 18 independent insertions all over the hslUV operon encoding one of the 5 major ATP-dependent proteases of E. coli and a homolog of the eukaryotic 26S proteasome (Coux et al. 1996). An hslUV over-expressing plasmid exaggerates the dnaA recBC co莱thality at 37°C (Fig. 2b), confirming suppression by hslUV inactivation. HslUV protease is proposed to degrade the unstable DnaA46 protein at higher temperatures (Katayama et al. 1996), offering the most likely mechanism of hslUV suppression (Fig. 2d).

We also isolated 4 insertions in and upstream of seqA gene as suppressors of the dnaA recBC co莱thality (Fig. 2a; Supplementary Fig. 1, a and b). The seqA suppressors were visibly stronger than the hslUV ones (Fig. 2, a and c). One of the seqA suppressors was tried and found to also suppress the co莱thality in the dnaA recBC strain that carries the hslUV-overexpressing plasmid (data not shown). The SeqA protein organizes E. coli chromosome in 2 important ways. SeqA forms filaments to organize the sister-chromatid cohesion zone behind the RF (Waldminghaus et al. 2012; Helgesen et al. 2015) via binding to hemi-methylated GATC sites (Brendler et al. 2000; Guarné et al. 2005). Equally important is SeqA binding at the hemi-methylated oriC, that negatively regulates replication initiation (Lu et al. 1994; von Freiesleben et al. 1994) by blocking long DnaA filament formation until oriC is fully methylated (Fig. 2d), thus setting the eclipse period, the minimal time between successive initiations (von Freiesleben et al. 2000; Olsson et al. 2002). This SeqA binding to the origin likely interferes with initiations by the partially active DnaA46 protein at higher temperatures; without SeqA, oriC will be available for DnaA polymerization any time (Fig. 2d).

We have also found that both the hslUV and seqA defects similarly suppress the dnaA601 recBC co莱thality combination and improve growth of dnaA601 at 39°C (Fig. 2c). However, in contrast to the dnaA46 mutant, the dnaA601 mutant is inhibited by additional copies of the mutant protein at 28°C (Hansen et al. 1992)—providing a likely explanation of the poor growth of the dnaA601 hslUV and especially of the dnaA601 recBC hslUV strains at this temperature (Fig. 2c). We also isolated a single insert upstream of dnaK, but this promising lead failed to confirm (see the Supplementary Results and Supplementary Fig. 1).

Suppressors of the dnaN recBC co莱thality

The dnaN recBC co莱thality was suppressed by 6 independent insertions in the iscSUA operon (Fig. 3a; Supplementary Fig. 2). The iscSUA operon and the hscB operon downstream are responsible for the synthesis and repair of iron-sulfur clusters that are used by key enzymes of the central metabolism (Vinella et al. 2009). We have also obtained insertions in the iscA gene which is a chaperone interacting with the IscU protein, as well as in pta, icd, and ubiC genes, all related to the central metabolism. As a result of their metabolic defects, all these suppressors grew slower (Fig. 3a, the 28°C plate).

Similar to isolating dnaA recBC suppressors in a major protease, 4 of our dnaN recBC suppressors had insertions in and upstream of the lon gene (Fig. 3a; Supplementary Fig. 2), which codes for another major ATP-dependent protease (Tsilibaris et al. 2006), suggesting that the DnaN(Ts) protein at the semipermissive temperatures is targeted by Lon. There was a single insert in the mismatch repair gene mutH, which we did not pursue further (Supplementary Fig. 2). Finally, one more insertion was in the dnaA gene, coding for a positive initiation factor, the DiaA protein, which assists ATP-DnaA polymerization at oriC for replication initiation (Fig. 2d) (Ishida et al. 2004). Indeed, a ΔdiaA allele rescues the dnaN recBC double mutant (Fig. 3b), strongly suggesting that the dnaN recBC co莱thality is caused by replication over-initiation.

Michel and colleagues have shown that the dnaN recBC double mutant undergoes RuvABC-dependent chromosome fragmentation, proposing this as an explanation for its co莱thality (Grompone et al. 2002). We inactivated the ruvA gene to test if blocking chromosome fragmentation suppresses the co莱thality, but found that the resulting dnaN recBC ruvA strain is still lethal at 37°C (Fig. 3c). Thus, prevention of RuvABC-dependent double strand DNA breaks at reversed RFs (Fig. 1b) does not rescue the dnaN recBC mutant.

Suppressors of the dnaC recBC co莱thality

The dnaC recBC co莱thality was suppressed by 3 inserts into and upstream of diaA (Fig. 3d; Supplementary Fig. 3), moreover, diaA deletion also rescues the dnaC recBC co莱thality (Fig. 3e). Thus, inactivation of the DnaA polymerization-promoting factor DiaA (Fig. 2d) suppresses both the dnaC recBC and dnaN recBC co莱thalities, strongly suggesting that the chromosome problems in both cases are caused by replication over-initiation.
We also found that insertions in both recR and recA genes suppress the dnaC recBC co lethality (Fig. 3d; Supplementary Fig. 3). The RecF, RecO, and RecR proteins work together in the RecA-mediated pathway to repair blocked single-strand DNA gaps (Kuzminov 1999), and expression of both recR and especially recA is elevated in the dnaC mutant at 38°C (Løbner-Olesen et al. 2008). It looks like DnaC malfunction causes formation of blocked single-strand gaps that apparently trigger recombinational misrepair or SOS induction by RecFOR—similar to what is proposed to happen during thymineless death (Fonville et al. 2010; Kuong and Kuzminov 2010). Alternatively, RecAFOR-initiated repair could also require DnaC-dependent loading of the replisome by PriA—while without replication restart, the unresolved daughter-strand gap repair intermediates generate problems that only
RecBCD-catalyzed repair can fix. The suppression by inactivation of the central activity of recombinational repair, the RecA protein, looks counterintuitive, but in fact makes sense, as there are only 2 recombinational repair pathways in E. coli cells, RecBCD and RecFOR, and both are already blocked in the suppressed dnaC recBC recR triple mutant.

Insertions inactivating the hssS gene and between the yjip and yjij genes also suppressed the dnaC recBC coletality (Fig. 3d; Supplementary Fig. 3). The latter insert is only 3 genes away from the dnaC itself and therefore could modulate dnaC expression. There were some more single suppressing insertions that we did not pursue further (Supplementary Fig. 3). Instead, we introduced the hsiUV suppressor of the dnaA recbc coletality into the dnaC recBC double mutant, but it failed to suppress (data not shown). And vice versa, the dnaA and recR suppressors of the dnaC recBC coletality failed to suppress the dnaA recBC coletality (Fig. 3f), highlighting the fact that even though both dnaA and dnaC are initiation functions, the nature of the dnaA recBC coletality is distinct from the dnaC recBC one.

Overall, suppressor analysis showed that synthetic lethality of the recBC defect with the replication initiation problems could have different nature, although some similarities were observed, too. In particular, suppression by protein degradation defects was expected for Ts-alleles, via stabilization of the mutant proteins at the semipermissive temperatures. Also, since both the dnaA recBC and dnaN recBC coletalities are suppressed by the dnaA defect, they must be caused by replication over-initiation, which is a known reason of coletality with the recBC defect in E. coli (Grigorian et al. 2003; Simmons et al. 2004; Rotman et al. 2014; Khan et al. 2016). In contrast, the dnaA recBC coletality is suppressed by the seqA defect and therefore must be caused by insufficient initiation (Fig. 2d), raising a possibility of a direct mechanistic RecBCD participation in the process of replication initiation. Thus, for the rest of the paper, we concentrate on the more mysterious dnaA recBC coletality, using the dnaC recBC and dnaN recBC coletalities in some physical assays as reference points.

Genetics of the dnaA recBC coletality

Recombinational repair is not required in the dnaA mutant

As mentioned in the Introduction, the RecBCD enzyme has 2 important roles in the cell: (1) degradation of linear DNA; (2) processing of a double-strand DNA end to prepare the substrate (3'-ss-end) for RecA polymerization, a prerequisite for recombinational repair. To test the role of recombinational repair in the viability of the dnaA mutant directly, we constructed the dnaA recA938 (null) double mutant. We observed that the recA defect shows only a weak interaction with the dnaA defect, further inhibiting, but not blocking, the growth of the double mutant (Fig. 4a). Likewise, introducing the recA938 defect in the recBC dnaA strain did not exacerbate the coletality beyond the expected growth inhibition (Fig. 4a). As a positive control for recA-dependence, we confirmed that the dnaN recA938 combination is even more coletal than the dnaN recBC one (Fig. 4a, bottom). Thus, the viability of the dnaA recA double mutant all but rules out that the dnaA mutant relies upon the recombinational repair function of RecBCD.

We have further tested the role of recombinational repair, separate from linear DNA degradation, using the plasmid pKD46 (Datsenko and Wanner 2000) into the dnaA ΔrecBCD strain. This plasmid, by supplying phage lambda recombination functions, significantly restores recombinational repair capacity to the ΔrecBCD mutant (Fig. 4b), without restoring linear DNA degradation capacity. We found that, though the strain dnaA ΔrecBCD pKD46 (+ ara) also shows enhanced UV survival (Fig. 4b) (suggesting partial restoration of recombinational repair), it still cannot grow at 39°C (Fig. 4c). Thus, the dependence of the dnaA mutant on RecBCD is not due to recombinational repair, suggesting that it is due to linear DNA degradation. And this switch from recombinational repair to DNA degradation dependence is clearly due to the dnaA mutation, as the difference in UV-survival between ΔrecBCD (ara) and the dnaA ΔrecBCD (ara) curves demonstrates.

DNA degradation by RecBCD is essential in the dnaA mutant

The above reasoning is further supported by the surprisingly strong lethality of the dnaA recCD combination (Fig 4d). The recD mutant is defective in the linear DNA degradation activity, but is fully recombination-proficient (Amundsen et al. 1986)—in fact, it is a hyper-rec in all homologous recombination assays, compared to WT (Chaudhury and Smith 1984; Lovett et al. 1988). This strong dnaA recD coletality confirms that normal recombinational repair capacity is not important for the dnaA mutant, while the normal linear DNA degradation capacity appears to be. The mutant essentially WT for recombinational repair yet completely deficient in linear DNA degradation is recBC shbc (Lloyd and Buckman 1985; Rao and Kuzminov 2019); in combination with the dnaA at 39°C, this mutant shows perhaps the strongest lethality reported in this work (Fig. 4e), suggesting that recombinational repair without linear DNA degradation becomes poisonous for the dnaA(Ts) mutant at the semipermissive temperature.

In order to conclusively determine which of the 2 general functions of RecBCD is required for survival of the dnaA mutants at 39°C, we introduced RecBCD mutant-carrying plasmids, which would restore either one of the 2 functions of the RecBCD complex—recombinational repair or DNA degradation, in the dnaA recBCD background (Miranda and Kuzminov 2003). Since the chromosomally expressed RecBC(Ts) protein could interfere with the RecBCD proteins supplied by the plasmids, we did this complementation in the dnaA ΔrecCD background (Fig. 4f). The low copy number plasmid (~6 per chromosome) introduces a mild overexpression of various RecBCD variants, which have no effect at 39°C in either ΔrecBCD or in dnaA single mutants (Supplementary Fig. 4). We found that introduction of either recombination function plus partial DNA degradation (Rec* Exo+), or full DNA degradation capacity without recombination (Rec− Exo+), was sufficient to restore the viability in the dnaA ΔrecBCD strain (Fig. 4f). We conclude that the dnaA strain requires linear DNA degradation function of the RecBCD protein for its survival, with no requirement for recombinational repair.

These requirements could be explained if, in the dnaA mutants, RFS stall and regress, yet do not break—because regressed RFS can be reset by RecBCD-dependent linear DNA degradation without repair, while broken RFS have to be reassembled by RecA-dependent recombinational repair (Fig. 4g).

The recG defect suppresses the dnaA ruvA coinhibition

To further guide our thinking, we introduced defects in HJ migration enzymes into our dnaA mutant. HJs, the 4-way fully duplex DNA junctions, form either during recombinational repair or by RF reversal (Fig. 1b, highlighted in yellow). Since the dnaA mutants do not depend on RecA, they do not need to resolve HJ after RecA-catalyzed recombinational repair; however, they may still need to move around HJs resulting from RF regress/reversal. Two activities handle HJs in E. coli (Kuzminov 1996). The RuvAB
DNA-junction-associated pump moves junctions around—so that the RuvC nuclease, that comes later and works at specific resolution sites, could split junctions in half by a pair of symmetrical nicks (Fig. 1b)—but the resulting disintegrated fork would require recombinational repair—which does not happen in our case. The second activity, the RecG dsDNA pump, is known to only push the junctions around. Interestingly, while both activities are required to remove HJs after recombinational repair, they seem to push the junctions in opposite directions (Fig. 5c), judging by their opposite phenotypes in some genetic systems (Foster et al. 1996; Harris et al. 1996).

It is important to mention that the recG defect, although only in combination with the tus mutation (inactivates replication termination sites) and a special rpoB" allele (alleviates replication-transcription conflicts), suppresses the dnaA46 initiation defect even at 42°C (Rudolph et al. 2013). Because of this, we did not test the recG inactivation in the dnaA recBC background, expecting either no effect or maybe a small growth improvement. Inactivation of HJ processing and resolution by the ruvA defect in the dnaA recBC strain did not rescue the strain (Fig. 5a), as was reported for the rep recBC colethals (Seigneur et al. 1998). In fact, the ruvA defect showed mild co lethality in combination with the dnaA defect alone (Supplementary Fig. 5, a and b), suggesting chromosomal problems in the dnaA mutant that require moving HJs, most likely RF regression. At the same time, the recG defect, that inactivates the second DNA junction Pushing activity (Kuzminov 1996), actually suppresses this synthetic inhibition of the dnaA recBC mutants (Fig. 5b), as if, after RF stalling, RecG and RuvA do move the resulting HJs in the opposite directions: RuvAB in the "right" direction, while RecG in the "wrong" one. However, recG defect (even alone) could ease the dnaA defect directly, via alternative initiations (Rudolph et al. 2013). At the same time, the lack of improvement of the dnaA recBC mutants poor survival at 39°C by inactivation of RuvA (in the dnaA recBC ruvA triple mutant) (Fig. 5a) shows that the chromosomal problems in dnaA recBC are not the result of HJ resolution by RuvC resolvase (which is made inactive by the ruvA defect).

Formulating a model

At this point, results of others and our own observations allowed us to formulate a model of chromosomal events in the dnaA46 mutant, to guide our subsequent inquiry. One requirement to
such a model was to explain the importance of linear DNA degradation by RecBCD and of moving around HJs by RuvAB vs RecG—both without subsequent recombinational repair (Fig. 1b or Fig. 4g). The second requirement was to explain how RecBCD-promoted degradation around these HJs facilitates an apparent release of DnaA from the chromosomal DNA to ensure new initiations at the origin, compensating for this mutant’s underinitiation.

We considered 2 specific facts about the dnaA46 defect: (1) in vitro, the mutant DnaA46 protein binds ~10 times more readily to oriC-containing DNA than WT DnaA protein does, at both low and high temperatures (Carr and Kaguni 1996); (2) due to defective DnaA46 autoregulation at higher temperatures, expression from the dnaA promoter region in the dnaA46 mutant is induced up to 4-fold upon shift from 30°C to 42°C (Atlung et al. 1985; Braun et al. 1985; Kücherer et al. 1986). Therefore, starting with the safe assumption that the mutant DnaA protein binds tighter (than WT DnaA) to its multiple sites on the chromosomal DNA in vivo and therefore stalls RFs at some of them, we propose that RecBCD-promoted linear degradation is needed to restart the stalled forks (Fig. 5c). Thus, the temperature-sensitivity of our dnaA(Ts) recBC(Ts) strain is not due to a tighter binding of the mutant DnaA protein to its chromosomal sites at 39°C [it binds tighter at any temperature (Carr and Kaguni 1996)], but due to 2 factors at higher temperatures: (1) the deficiency in linear DNA degradation of our recBC(Ts) mutant; (2) the increased expression of the auto-regulated DnaA(Ts) protein. One obvious prediction from the idea—since the dnaA46 mutant at any temperature depends on reset of stalled RFs, it should be synthetic lethal, even at 30°C, with defects in RF restart—was already tested: the dnaA46 priC mutant is indeed synthetic lethal at 30°C (Hinds and Sandler 2004).

Specifics of the model include explanation of the ruvA and recG effects, as well as how recombinational repair is avoided. Since in the stalled forks the replisomes are still intact, such stalled forks will be hard to reverse and so need RuvAB’s help to do so, while RecG would catalyze reverse branch migration (Whitby et al. 1993), converting the resulting HJs back to RFs, which is counter-productive (Fig. 5c). We further postulate that, even though the RFs are eventually reversed, they are not split by RuvC resolution, because such splitting would make it all dependent on recombinational repair (Fig. 1b or Fig. 4g), which we do not observe. The independence of recombinational repair of the dnaA mutant also suggests that the generated linear tails at reversed forks are relatively short (much shorter than ~5 kb) and therefore do not have a chance to initiate repair at Chi-sites, as the longer tails would—otherwise recombinational repair would have had an effect. Chi-sites are 8bp-long special sequences, found in the E. coli chromosomal DNA once per ~5 kb, at which progressing RecBCD switches from the DNA degradation mode to the DNA repair mode (Myers and Stahl 1994). The short linear tail is simply degraded by RecBCD, which resets the fork (Fig. 5c). The fork resetting event likely recruits the Rep helicase as the booster motor for the replicative helicase DnaB (Guy et al. 2009; Atkinson, Gupta, and McGlynn 2011), which allows the “turbo” fork to disperse the DnaA barrier, then Rep dissociates. In the cell, Rep associates with the replisomes only transiently, but at any given moment colocalizes with 70% of RFs (Syeda et al. 2019). Overall, the action of RecBCD and Rep makes RFs more processive.

Thus, according to the model, RecBCD does not release DnaA from the chromosome directly by degrading long linear DNA tails; rather, steady DnaA release from the chromosomal DNA is promoted by processive replication, supported by RecBCD and Rep. Mechanistically, the DnaA release by progressing RFs in E. coli is likely the function of the Hda protein, which tightly associates with the DNA clamp within active replisomes (Kawakami et al. 2006). Furthermore, we imagine that, at the chromosomal level, the increased processivity of RFs allows DnaA redistribution from the chromosomal binding sites to the oriC polymerization site (Fig. 5d). The interesting corollary of such thinking is that not only replication elongation is expected to be inhibited in the dnaA recBC mutants, but also new initiations would be blocked, due to the general unavailability of DnaA (Fig. 5d).

The model generates other testable predictions: (1) any manipulation with the copy number of the major ATP-DnaA hydrolysis site datA should exacerbate dnaA recBC co lethality (as explained below); (2) as a tighter DNA binder, the DnaA46 mutant protein, especially if overproduced, should inhibit growth of dnaA46 cells; (3) the auxiliary helicase Rep should be required for survival in the dnaA mutants; (4) chromosomal fragmentation...
should be minimal in the dnaA recBC mutant—mostly suggested by lack of need for recombinational repair; (5) in the dnaA (RecBCD\(^{-}\)) cells at 39°C, the overall replication will be inefficient, due to the complicated mode of conflict resolution, while in the dnaA recBC mutants at the 39°C, the replication will be severely inhibited, both at the level of elongation and initiation. Below we tested these predictions.

**Testing the model of the dnaA recBC colethality**

**Effect of the datA deletion or multiple copies of datA**

The copy number of DnaA is constant and is about 1,000–2,000 copies per rapidly growing cell (Sekimizu et al. 1988), distributed in normally growing cells between \(~20\%\) ATP-DnaA (efficient DNA binding) and \(~80\%\) ADP-DnaA (inefficient DNA binding) (Kurokawa et al. 1999). Chromosome distribution of the DNA-bound DnaA is dynamic and maintained at its optimum by the ATP-DnaA to ADP-DnaA ratio. There are 2 processes (due to complex names known by their acronyms) that convert ATP-DNA to ADP-DnaA, thus promoting DnaA redistribution: RF (Hda)-dependent “RIDA,” happening at the origin and throughout the chromosome (Kato and Katayama 2001), and (IHf)-dependent “DDAH,” happening continuously at the datA chromosomal locus (Kasho and Katayama 2013). There are also 2 processes that continuously recharge DnaA with ATP: one operates at 2 DnaA-reactivation sequences in the chromosome (Fujimitsu et al. 2009), while the other uses membrane phospholipids (Garner and Crooke 1996).

If the main problem of the dnaA recBC colethality is inadequate accumulation of the mutant DnaA protein at the origin due to the tight DnaA46 binding to its chromosomal sites (in other words, RIDA is underperforming in the dnaA46 mutant), this problem will be exacerbated by upsetting the ATP-DnaA to ADP-DnaA ratio in either direction. Inactivation of DDAH by deleting datA should further increase the ATP-DnaA fraction, thus slowing down the ATP hydrolysis-stimulated redistribution among its chromosomal sites. This is expected to further exacerbate the problem of the mutant with a tighter-DNA-binding DnaA, which, as we postulate, already suffers from the inefficient redistribution rate by increasing the fraction of ATP-DnaA, thereby decreasing initiation by decreasing the fraction of ATP-DnaA, again does (Fig. 6b). Thus, either decreasing the DnaA chromosome redistribution rate by increasing the fraction of ATP-DnaA, or decreasing initiation by decreasing the fraction of ATP-DnaA, both exacerbate the dnaA recBC colethality—and both are consistent with the primary defect being tighter mutant DnaA binding to its multiple chromosomal sites.

**Overexpressed DnaA46 protein inhibits dnaA+ cells**

According to our model, overproduction of the DnaA46 mutant protein should interfere with growth of dnaA\(^{+}\) cells. To test this prediction, we cloned the WT dnaA and dnaA46 genes in pMTL20 (Chambers et al. 1988) and introduced these constructs in AB1157, recBC, dnaA, and dnaA recBC strains (Fig. 6c; Supplementary Fig. 5). Overexpression of the DnaA46 protein was toxic for all strains at 28°C, this toxicity being somewhat relieved at higher temperatures of 37, 39, and 42°C, especially in the dnaA single mutant (Fig. 6c). The inhibitory effect of overproduction of DnaA46 protein and similar DnaA(Ts) mutant proteins at lower temperatures in dnaA+ cells was noted before (Hansen et al. 1992; Carr and Kaguni 1996; Nyborg et al. 2000). High copy number of DnaA46 was able to completely rescue the matching dnaA mutant at the higher temperatures, while allowing the double dnaA recBC mutant to grow (slowly) at all these temperatures (Fig. 6c).

In other words, increasing the concentration of defective DnaA46 compensates for the defect in its function at higher temperatures—consistent with HslU UV protase inactivation suppressing the dnaA recBC colethality (Fig. 2). Overproduction of several DnaA(Ts) proteins compensates for the temperature-sensitivity of the correspondent mutants (Hansen et al. 1992).

Interpretation of these results is somewhat complicated by the fact that our high-copy number vector (pMTL20) itself interfered with growth of the dnaA+ strains at these higher temperatures, while the same plasmid, but carrying dnaA46 gene, improved the growth at these temperatures (Fig. 6c). Perhaps this effect is due to the presence of a single DNA binding site on pMTL plasmids, but we have not tested this idea. This vector interference with growth at 42°C could be again observed with strains carrying WT dnaA constructs, but in this case, overexpressing WT dnaA was only mildly inhibitory at 28°C and allowed all 4 strains to plate at full titer at 42°C (Supplementary Fig. 5).

**The dnaA recBC requires Rep even at 28°C**

As explained in the introduction, the overall RF movement is slower in the rep mutants due to frequent pauses (Lanef and Denhardt 1975; Guy et al. 2009; Atkinson, Gupta, and Rudolph, et al. 2011), while in vitro the Rep helicase boosts the DnaB motor to plough RFs through obstacles in the template DNA (Guy et al. 2009; Atkinson, Gupta, and McGlynn 2011). If the DnaA46 protein clusters bound to DNA indeed represent a hindrance to RFs, the activity of the Rep helicase should become essential in the dnaA recBC mutant at lower temperatures. We have found that at 28°C, the rep dnaA recBC triple mutant is severely inhibited, compared to the rep dnaA or rep recBC double mutants, and is completely dead at 35°C, suggesting increased impediments ahead of forks (Fig. 6d). The rep recBC double mutant is itself synthetic lethal at 37°C [nonpermissive for recBC(Ts)] (U zest et al. 1995) and is inhibited for growth even at lower temperatures (Fig. 6d), demonstrating that the weakened activity of mutant RecBCD compromises survival of cells that cannot power-up their RFs. This is also substantiated by the more severe inhibition of the rep mutant by overexpression of DnaA46, compared with WT dnaA at 28°C (Supplementary Fig. 6). Expectedly, the DnaA46-carrying plasmid is tolerated better by this strain at higher temperatures, while the WT dnaA-carrying plasmid shows no temperature effect (Supplementary Fig. 6).

**The dnaA defect does not cause chromosome fragmentation**

A colethality of a particular mutation with the recBC defect usually indicates formation of double-strand breaks as a result of interference with DNA replication (Kuzminov 1999; Michel et al. 2001). The 2 major endogenous reasons for RF instability causing double-strand breaks are (1) increased spontaneous DNA damage and intermediates of its excision repair; (2) general inhibition of RF progress (Fig. 1b). It is the second reason that could potentially result in chromosomal fragmentation in the dnaA mutants. A useful characteristic of the recBC mutants is quantitative “preservation” of their levels of chromosomal fragmentation—because these mutants neither repair double-strand breaks, nor degrade linear DNA (Michel et al. 1997). We compared the 3 synthetic lethal strains: dnaA recBC, dnaA recBC, and dnaC recBC at the non-permissive temperatures for their recBC(Ts) alleles for their levels of chromosomal fragmentation (Fig. 6, e and f).
For practical reasons, at this point we had to switch from plating, convenient for viability studies, but taking days to develop—
to liquid cultures, which facilitate physical studies over shorter
time spans. For example, chromosome fragmentation is typically
calculated after 4 h at the nonpermissive temperature
(Kouzminova et al. 2004). We found that dnaN recBC strain shows
\( \Delta C24 C16\% \) chromosomal fragmentation above the background at
37°C (Fig. 6, e and f), but little fragmentation at 39°C (data not shown, was also reported before (Grompone et al. 2002)),
while the dnaC recBC strain shows \( \Delta C5\% \) chromosome fragmentation
over the background at 37°C (Fig. 6, e and f) and similar numbers
at 39°C (data not shown)—which would likely become significant
with more repetitions. At the same time, we could not detect any
statistically significant fragmentation in the dnaA recBC strain
over the dnaA single or recBC single mutant background, at either
37°C (Fig. 6, e and f) or 39°C (data not shown), indicating that the
dnaA defect does not result in appreciable number of breaks at
both RFs at the same time. The undetectable chromosome frag-
mentation in the dnaA mutant is a deviation from a typical be-
havior of RecBCD-dependent mutants observed so far, but is
predicted by our model of the dnaA recBC defect (Fig. 5c) and is
consistent with its complete independence of recombinational
repair (Fig. 4).

Both replication initiation and existing fork elongation are blocked in the dnaA recBC double mutant
Our viability studies show that the DNA degradation activity of the RecBCD enzyme is essential for the dnaA mutant at the semi-
permissive temperature, most likely facilitating replication, but it
was unclear which replication stage was more affected, initiation
or elongation. To identify the most affected replication stage in
the double dnaA recBC mutant at 39°C, we followed the time
course of accumulation of the chromosomal origin signal vs the
terminus signal, comparing the double mutant to the corre-
sponding single mutants (the recBC mutant in this test behaves
essentially as WT) (Fig. 7a and b). Thus, we compared replication
initiation (by quantifying oriC) with replication elongation (by quantifying the ter region) (Rao and Kuzminov 2019), in the deep
dilution conditions (Fig. 1e).
The single recBC mutant showed robust initiation at 39°C: the 16-fold increase in oriC within the first hour (Fig. 7a) indicates 4 initiation rounds. The replication elongation in the recBC mutant, indicated by the matching terminus increase, was equally robust (Fig. 7b). In contrast, dnaA and recBC dnaA cells show a single initiation event at 39°C, after which dnaA recBC stops further initiations, while dnaA makes another one by 2 h and hints at another “half-round” by 4 h (Fig. 7a). At the same time, existing RFs in the dnaA single and dnaA recBC double mutants are operational, although inhibited compared with recBC, as the increase in the terminus quantity indicates (Fig. 7b). In particular, the dnaA recBC mutant manages to finish 2 replication rounds by 2 h before plateauing, while dnaA makes another one by 2 h and also pauses, but seems poised to resume replication after 3 h (Fig. 7a). At the same time, existing RFs in the dnaA single and dnaA recBC double mutants are operational, although inhibited compared with recBC, as the increase in the terminus quantity indicates (Fig. 7b). In particular, the dnaA recBC mutant manages to finish 2 replication rounds by 2 h before plateauing, while dnaA makes another one by 2 h and also pauses, but seems poised to resume replication after 3 h (Fig. 7a). In general, however, the 2 mutants show little difference in their degree of initiation inhibition and slower elongation progression within the first 3 h at 39°C, suggesting that the RecBCD effects start later.

We confirmed that this broad replication inhibition is the primary defect in the dnaA mutant by doing the same origin and terminus copy number time course analysis in the 2 dnaA recBC colethality suppressors, seqA and hslUV. We observed that both suppressors significantly improved origin initiation in the mutant (Fig. 7c), while also improving replication elongation in the seqA mutant, but not much in the hslUV mutant (Fig. 7d), consistent with a better growth of the dnaA recBC seqA compared to the dnaA recBC hslUV (Fig. 2, a and c). In fact, elongation inhibition was expected in the hslUV suppressor, because in the absence of the HslUV degradation, the increased copy number of the DnaA46 protein should increase its overall binding to the chromosome.

In contrast with the dnaA recBC colethal, the dnaN recBC colethal shows a milder replication defect, which appears no different than in the single dnaN mutants (Fig. 7, e and f). In particular, the origin increase in the first 2 h is the same for the 3 strains (recBC, dnaN, and recBC dnaN) and only then becomes slower in the dnaN mutants (Fig. 7e). The terminus increase in both dnaN mutant strains is more modest (Fig. 7f), reflecting the expected elongation problems. Looking closer, it becomes apparent that the 2 curves (for origin and terminus) are almost superimposable for the dnaN single mutant (compare Fig. 7, e vs f), suggesting a modest elongation defect. In contrast, in the dnaN recBC mutant, the origin increase curve was twice as steep as the terminus increase one (compare Fig. 7, e vs f), indicating significant elongation problem without RF repair in the dnaN mutant. This finding is in agreement with the significant chromosome fragmentation in this mutant (Fig. 6, e and f). Overall, we conclude that the dnaA recBC mutant stops initiations and slows down elongation at the nonpermissive temperatures, while the dnaN recBC mutant at first initiates normally at the nonpermissive temperature, but soon runs into problems with elongation, and its initiation also becomes affected.

**RecBCD restarts RFs frozen in the dnaA mutant**

Puzzled by the apparent lack of RecBC effect for chromosomal replication in the dnaA and dnaN mutants (Fig. 7), we measured a
different replication parameter, and for longer times. To see whether RecBCD works continuously to support new initiations in the dnaA mutant, or reacts to initiation problems only after they arise, we used 3H-dT incorporation to compare the rate of DNA replication in the dnaA single vs dnaA recBC double mutant cultures at various temperatures (Fig. 8). For practical reasons, the initial experiments were done with undiluted cultures, when the viability loss of the double mutant was minimal (Fig. 1d).

The 4 strains (the WT, the 2 single mutants and the double mutant) were grown at 28°C till they reached OD = 0.1, at which time (T = 0) we started measuring their DNA synthesis rate at regular intervals, while the strains were continued at 28°C (Fig. 8a). After 60 min at 28°C (at which point all 4 strains still showed comparable DNA synthesis rates), the 4 cultures were shifted to 39°C for 120 min, then brought back to 28°C for another 60 min (Fig. 8a).

The wild-type and the recBC single mutant showed comparable and high rates of DNA synthesis at either 39°C or later at 28°C, although half-way through 39°C the rates stabilized, indicating exit out of logarithmic growth (Fig. 8a). Also, the recBC(Ts) mutant significantly reduced the rate after this stabilization at 39°C, likely because of its inability to reassemble disintegrating RFs at this temperature. In contrast, the dnaA single and the dnaA recBC double mutants showed an initial brief spike after 15' at 39°C, followed by gross inhibition of DNA synthesis rates after 30' at 39°C (Fig. 8a). Thus, our DNA synthesis rate measurements supported the earlier conclusion about inhibited elongation combined with deficient initiation in the dnaA(Ts) mutant after shifting to 39°C. The DNA synthesis rates in the dnaA recBC double mutant remained flat while the strain was at 39°C, recovering only when the strain was returned to 28°C. In contrast, the dnaA single mutant gradually resumed replication at 39°C, revealing the action of RecBCD, but reaching WT levels only after return to 28°C (Fig. 8a).

To make sure our observations in standard-dilution cultures were robust, we also measured DNA synthesis rate in deeply diluted cultures, in which the double mutant dies (Fig. 1e), incubating them at 39°C for 7 h straight. Under these more stringent conditions, the rate of DNA synthesis in the dnaA recBC double mutant never recovered above the background, while the rate in the dnaA single mutant did eventually recover, but only after a 3-h lag (Fig. 8b), validating the earlier hint of recovery (Fig. 7, a and b). Thus, the RecBCD enzyme does not act continuously in the dnaA(Ts) mutant at the semi-permissive temperature, but eventually starts facilitating replication, both the initiation and elongation stages.

![Fig. 8. The rate of DNA synthesis. In Y-axis, CPM = “counts per minute.” a) The rate of DNA synthesis in the indicated strains at the indicated temperatures. At the time 60’, there was a 28°C → 39°C shift-up, while at 180’, there was a 39°C → 28°C shift-down. The cultures were not diluted. The average background ± SEM is shown as the gray line. b, c) The rate of DNA synthesis of the indicated mutants at the indicated semi-permissive temperatures for single dna mutants, in ×30 diluted cultures. d) Interpretation of the replication defects of the dnaA versus dnaN mutants and their “repair” by RecBCD.](image-url)
The situation was again quite different in the dnaN vs dnaA recBC mutant at 37°C (their semi-permissive temperature) (Fig. 8c): although both mutants kept similarly reduced levels of replication at the semi-permissive temperature for 2 h, the dnaN single mutant then continued with a brief exponential increase, while the dnaN recBC double mutant leveled off and reduced its replication, indicating elongation crisis. Thus, in the dnaN(Ts) mutant at 37°C, RecBCD clearly supports the integrity of RFs (Fig. 8d).

Discussion

The recBCD defect in the major double-strand break repair and linear DNA degradation activity of E. coli is synthetic lethal with various other defects in the DNA metabolism, for example, dut, nthAB, or ligA—rationalized by the increased frequency of double-strand DNA breaks in these mutants (Kouzminova and Kuzminov 2004, 2012; Kouzminova et al. 2017). In all the cases, massive chromosome fragmentation is readily detectable in the double mutants and requires active RFs. RFs in general are the most vulnerable chromosomal points—and RecBCD-dependence of various replication elongation mutants provides ample confirmation of this concept (Michel et al. 1997; Flores et al. 2001; Grompone et al. 2002). At the same time, defects in replication initiation were not expected to lead to double-strand DNA breaks—for the simple reason that they tend to align the chromosomes (making them free of RFs), by allowing existing replication rounds to complete and at the same time blocking new initiations. Therefore, our original finding that the dnaA and dnaC defects in replication initiation are synthetic lethal with the recBC defect (Fig. 1) appeared to make no sense. Suppressor analysis revealed an unexpected complexity, as the 2 mutants were recBC-colethal for opposite reasons: too much initiation in the dnaC mutants, while too little in the dnaA one. We decided to concentrate on the dnaA mutant defect, as the one without an obvious explanation, but with a hint at the possibility of a replication initiation role for the RecBCD enzyme.

We found that the dnaA mutant relies on the DNA degradation function of RecBCD, with either undetectable or a slightly poisonous role of recombinational repair (Fig. 4), but some roles for HJ migration (Fig. 5). Moreover, suppressors of the dnaC recBC colethality point to insufficient initiation, suggesting that there is not enough initiation-competent DnaA(Ts) protein, while linear DNA degradation somehow compensates for the defect, and HJs are involved. Therefore, we proposed that the mutant DnaA protein (known to bind DNA more readily than WT DnaA [Carr and Kaguni 1996]) has problems dissociating from its multiple chromosomal binding sites and thus stalls RFs (Fig. 5c). The stalled RF reversal, followed in RecBCD+ cells by linear tail degradation, resets the forks, while transient recruitment of Rep makes the reset forks powerful enough to break through the DnaA barriers (Fig. 5c). Steady progress of such “turbo” RFs allows not only to keep replication elongation going, but also to displace from the template DNA enough DnaA for initiation of new replication rounds (Fig. 5d). But this RF reset is disabled in the recBC(Ts) mutant at 37°C and higher, due to RecBCD inactivation.

Testing various predictions of this model yielded supporting results (Figs. 6–8), even though for physical studies we had to switch from “several day incubation on plates” time frame to “several hours in liquid cultures” time frame. In particular, it was found that the dnaA recBC mutants do not fragment their chromosome (Fig. 6, e and f) and that replication elongation and initiation are both completely blocked in the mutant (Figs. 7 and 8). We conclude that linear DNA degradation by RecBCD compensates for the chromosomal defects of the partially active DnaA mutant protein by resetting and thus “invigorating” the inhibited RFs.

The proposed tighter DNA binding by the mutant DnaA in vivo appears to be directly testable by whole-cell methods like ChIP-seq or Gfp-DnaA imaging. However, further examination of this idea makes the differences hard to detect, since the mutant protein is expected to bind the same chromosomal sites and at the same general copy number as the WT DnaA. The only proposed difference is the rate of dissociation upon RF arrival, which would be hard to see by methods looking at the entire chromosome. In the absence of obvious tests of this idea in vivo, we have to broaden possible causative defects of the DnaA(Ts) protein to include such formal alternatives as decreased nucleotide binding at higher temperature, decreased ATP-dependent filament formation at oriC or increased protein aggregation.

Oddities

In this section we briefly touch on several unusual observations without obvious explanations. The density-dependent survival of the double mutant (Fig. 1, d and e) could be easily explained if related to cellular scavenging systems, like catalases, which do offer better protection at higher cell densities—but nothing of this kind is known for either DnaA or RecBCD mutants. In general, density-dependent survival probably reflects the presence of various components of the quorum sensing systems in E. coli (Ahrner 2004).

Although RuvAB and RecG are analogs in that they both migrate Holiday junctions along the DNA molecules (Lloyd and Sharples 1993; Eggleston et al. 1997), and the corresponding mutants both decrease homologous recombination or survival after DNA damage (Lloyd 1991), the 2 mutants have the opposite phenotypes in our genetic system: ruvAB inhibits dnaA(Ts) growth at the semi-permissive temperature, while recG enhances it (Fig. 5, a and b). There are only a few reports of the ruvAB and recG defects acting in opposite directions, and in all of them the ruvAB defect lowers the WT numbers, while the recG defect increases them. One such genetic system is deletion between tandem repeats in plasmids (Lovett et al. 1993), another is recombinational repair of a chromosomal double-strand cut off a partially homologous plasmid (Grove et al. 2008), and perhaps the best known one is the “adaptive mutagenesis” (Foster et al. 1996; Harris et al. 1996). The disparate effects of the 2 defects in HJ-moving enzymes, as well as the difference in their genetic suppressors, support the idea that their cellular functions, once postulated for both to be processing of recombinational repair intermediates (Lloyd 1991; Lloyd and Sharples 1993), are actually quite different: removal of HJs for RuvABC, while preventing over-replication after recombinational repair for RecG (Zhang et al. 2010). It is, in fact, this “overreplication,” which allows the recG defect to suppress the dnaA(A6) defect at the nonpermissive temperature of 42°C, in the special Δrus rpoB*35 background (Rudolph et al. 2013).

Dependence of a particular mutant on RecBCD does not always mean that the mutant is equally dependent on recombinational repair (RecA). Although similar RecA- and RecBCD-dependence is typical (Kouzminova et al. 2004), there are mutants, like rep (auxiliary replicative helicase) or nthA (RNase HI), that depend more on linear DNA degradation by RecBCD and less on recombinational repair—these mutants can survive recA inactivation (even though they are usually inhibited by RecA loss) (Itaya and Crouch 1991; Uzest et al. 1995). At the same time, the
The phenomenon of synthetic lethality (co lethality) (Guarente 1993; Nijman 2011), frequently comes as a surprise, although it should be expected. Logically, if there is a significant function, inactivation of which leads to cell inviability [and there are hundreds of essential functions (Gerdes et al. 2003)], there could be two different enzymes in the same cell performing this same function (Ting et al. 2008), providing the level of “buffering via redundancy” the biological systems are famous for (Tautz 1992; Wilkins 1997; Hartman et al. 2001). In fact, this natural explanation of synthetic lethality as a combination of two mutations inactivating the alternative branches of the same essential pathway is still the most intuitive one (Guarente 1993; Hartman et al. 2001; Ooi et al. 2006).

However, as argued before, the majority of synthetic lethal combinations are avoidance-repair couples, in which one defect allows a potentially lethal problem to arise frequently, while the other defect blocks the repair of this problem (Ting et al. 2008; Mahaseth and Kuzminov 2017).

The lethality of dnaA recBC combination cannot be due to redundancy for obvious reasons, but is it the “avoidance-repair couple” type? In such a couple, RecBCD would be, naturally, the repair activity, while the DnaA would have to be the avoidance activity. But what DNA lesion is repaired or avoided in this case? If the repair enzyme is RecBCD, its only known substrates are double-strand DNA breaks (Dillingham and Kowalczykowski 2008), and these are not detected in the double mutant (Fig. 6, e and f). What is clear from our physical analysis is that RecBCD acts to help DnaA mutant protein to perform its activity—the initiation of chromosomal replication at oriC. From this perspective, the dnaA recBC olethality looks like another case of “compensation,” when a hypomorph mutant of an essential function is operational as long as it is supported by other cellular activities; when this support is withdrawn by additional mutations, the hypomorph fails in its function. For example, the temperature-sensitivity of dnaA46 mutant is suppressed in pta and ackA mutants that accumulate acetate and pyruvate (Tynecka-Mulik et al. 2017), suggesting mutant protein compensation by cytoplasmic chemistry.

The idea of compensation is analogous to the concept of “biochemical buffers” explaining how HSP90 chaperone activity essentially hides the genetic diversity of tumors, catalyzing their phenotypes (Whitesell and Lindquist 2005). In fact, overproduction of GroEL/GroES, the major protein chaperone in E. coli, relieves temperature-sensitivity of the dnaA46(Ts) mutant (Fayet et al. 1986), demonstrating compensation in action. Obviously, compensation as a co lethality phenomenon is possible only when one of the two lethal mutations is a hypomorph—and both the dnaA(Ts) and the recBC(Ts) defects in this case fulfill this requirement. We have encountered cases of compensation co lethality before with the dut-1 defect, also a hypomorph of an essential gene (Ting et al. 2008). However, the dnaA recBC case cannot be compensation only, because the dnaA defect does lead to a chromosomal problem (inhibited replication, HJs), which, in the form of regressed RFs, is “repaired” by RecBCD-promoted degradation—thus, indirectly arguing for the avoidance-repair couple relationship. We propose that dnaA recBC is a hybrid case of synthetic lethality that has elements of both the avoidance-repair couple and compensation.

**Conclusion: the DnaA-chromosome balance**

RecBCD, as the most powerful helicase/nuclease of bacterial cells, was always considered a major supporting function for replication elongation, ensuring the steady progress of RFs via linear DNA degradation at regressed forks or via RecA-catalyzed recombinational repair of disintegrated forks (Kuzminov 1999, Dillingham and Kowalczykowski 2008). Here, we describe the situation, in which RecBCD definitely supports replication initiation, even though indirectly, by performing its main function, ensuring progress of RFs. In other words, the steady RF progress is important for the timely initiation in the dnaA mutant, reflecting the complex interplay between elongation and the regulation of new initiation rounds, achieved via the DnaA protein binding to, and displacement from, the chromosomal DNA. DnaA binds to more than 300 sites throughout the chromosome (Hansen and Atlung 2018), but can polymerize only at oriC and a few other sites (Roth and Messer 1998). The balance between the general chromosome binding vs oriC polymerization is apparently upset in our dnaA(Ts) mutants in favor of the chromosome, making steady RF progress a prerequisite for rebalancing. Overproduction of some DnaA(Ts) proteins were reported to inhibit replication elongation at lower temperatures (Nyborg et al. 2000). Thus, dnaA recBC colethality highlights the chromosomal aspect of the regulation of replication initiation. There is also the cell growth aspect, pertaining to the copy number of DnaA per cell, which was not monitored in our study.
Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplemental material is available at G3 online.

Acknowledgments

We are grateful to Sharik Khan (this laboratory) for the original finding of the dnaA recBC co lethality and for his generous help at the beginning of this project, to Beronda Montgomery (Michigan State University) for guidance with the early version of the paper and to Lenna Kouzmanova (this laboratory) for critical reading of the final version. We would like to thank the entire Kuzminov laboratory for encouragement and help with this project.

Funding

This work was supported by the grant GM 132484 from the National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflicts of interest

None declared.

Supplemental material

Supplemental material is available at G3 online.

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