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RESEARCH ARTICLE

Topoisomerase I Essentiality, DnaA-Independent Chromosomal Replication, and Transcription-Replication Conflict in *Escherichia coli*

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ABSTRACT Topoisomerase I (Topo I) of *Escherichia coli*, encoded by *topA*, acts to relax negative supercoils in DNA. Topo I deficiency results in hypernegative supercoiling, formation of transcription-associated RNA-DNA hybrids (R-loops), and DnaA- and *oriC*-independent constitutive stable DNA replication (cSDR), but some uncertainty persists as to whether *topA* is essential for viability in *E. coli* and related enterobacteria. Here, we show that several *topA* alleles, including Δ*topA*, confer lethality in derivatives of wild-type *E. coli* strain MG1655. Viability in the absence of Topo I was restored with two perturbations, neither of which reversed the hypernegative supercoiling phenotype: (i) in a reduced-genome strain (MDS42) or (ii) by an RNA polymerase (RNAP) mutation, *rpoB*35*, that has been reported to alleviate the deleterious consequences of RNAP backtracking and transcription-replication conflicts. Four phenotypes related to cSDR were identified for *topA* mutants: (i) one of the *topA* alleles rescued Δ*dnaA* lethality; (ii) in *dnaA*$^+$ derivatives, Topo I deficiency generated a characteristic copy number peak in the terminus region of the chromosome; (iii) *topA* was synthetically lethal with *rnhA* (encoding RNase HI, whose deficiency also confers cSDR); and (iv) *topA rnhA* synthetic lethality was itself rescued by Δ*dnaA*. We propose that the terminal lethal consequence of hypernegative DNA supercoiling in *E. coli topA* mutants is RNAP backtracking during transcription elongation and associated R-loop formation, which in turn leads to transcription-replication conflicts and to cSDR.

IMPORTANCE In all life forms, double-helical DNA exists in a topologically supercoiled state. The enzymes DNA gyrase and topoisomerase I act, respectively, to introduce and to relax negative DNA supercoils in *Escherichia coli*. That gyrase deficiency leads to bacterial death is well established, but the essentiality of topoisomerase I for viability has been less certain. This study confirms that topoisomerase I is essential for *E. coli* viability and suggests that in its absence, aberrant chromosomal DNA replication and excessive transcription-replication conflicts occur that are responsible for lethality.

KEYWORDS topoisomerase I, R loops, constitutive stable DNA replication, transcription-replication conflict, DNA supercoiling

DNA in all cells is negatively supercoiled, and in bacteria such as *Escherichia coli*, two enzymes, gyrase and topoisomerase I (Topo I), ordinarily act oppositely to maintain the homeostasis of DNA superhelical density (reviewed in references 1 to 4). DNA gyrase is a hetero-tetrameric enzyme (comprised of two subunits each of GyrA and GyrB proteins) that is ATP dependent and introduces negative supercoils, whereas Topo I (encoded by *topA*) is an 865-amino-acid monomer that catalyzes relaxation of supercoiled DNA in an energy-independent reaction. An important role for Topo I is in the dissipation of negative supercoils, in accord with the twin-domain supercoiling

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model (5, 6), that are generated behind RNA polymerase (RNAP) in the transcription elongation complex (TEC).

That gyrase deficiency leads to bacterial death is well established. On the other hand, the essentiality of Topo I for viability, in either *E. coli* or closely related bacteria such as *Salmonella enterica* and *Shigella flexneri*, is somewhat less certain (7–13). One difficulty has been that *topA* mutants rapidly accumulate suppressors which are often in the genes encoding the gyrase subunits (8–10, 13–17), and consistent with their opposing actions, gyrase and Topo I mutations can, in combination, partially cancel one another's sickness or inviability (18, 19). Growth of *E. coli topA* mutants is also improved upon overexpression or amplification of genes encoding the topoisomerase III (13, 18, 20) or IV (10, 16, 19).

Topo I deficiency is associated with an increased prevalence of R-loops (RNA-DNA hybrids) in the cells, which has been attributed to reannealing of the 5′ end of nascent RNA into hypernegatively supercoiled DNA behind the TEC under these conditions (21–23; for reviews, see references 4, 24, and 25). Overexpression of RNase HI (encoded by *rnhA*), which degrades RNA in RNA-DNA hybrids, can alleviate some of the phenotypes of *topA* mutants (18, 22, 23, 26–28), and conversely, *topA rnhA* mutants exhibit exacerbated sickness (13, 26, 29). In principle, R-loops can exert toxicity by acting as roadblocks to subsequent transcription (30, 31) and to replication (32–34); a third mechanism for toxicity is by serving as sites for initiation of aberrant chromosomal replication, as further outlined below. That R-loop formation is modulated by DNA supercoiling has been shown also in the CRISPR-Cas9 system (35) and in eukaryotic cells (36–38).

Recent evidence indicates that transcription-replication conflicts can themselves lead to increased formation of R-loops in the genome following RNAP backtracking at the sites of conflict (39–42; for reviews, see references 43 to 45). It has also been suggested that extended RNAP backtracking could be associated with R-loop formation from the 3′ end of the nascent RNA (40, 46).

R-loops are physiological initiators of ColE1 plasmid replication (47), but in addition, their excessive occurrence (as in *rnhA* mutants) can lead to pathological initiation of chromosomal DNA replication in both bacteria (reviewed in references 48 to 50) and eukaryotes (51). Such aberrant replication in bacteria is referred to as constitutive stable DNA replication (cSDR) since, unlike ordinary chromosomal replication, which is initiated at *oriC* with the aid of the unstable protein DnaA, it continues long after inhibition of protein synthesis in the cells. cSDR can be identified biochemically as persistent DNA synthesis following addition of translational inhibitors such as chloramphenicol or spectinomycin.

cSDR can also be identified genetically as rescue of lethality associated with loss of DnaA function, which is a more stringent test of cSDR, since it demonstrates the capability to duplicate the entire chromosome in the absence of *oriC*-initiated replication (48, 49). During its progression around the bacterial chromosome, such aberrant replication would be expected also to encounter (i) increased head-on conflicts with TECs on heavily transcribed genes (especially the *rrn* operons) that have evolved to be codirectional with *oriC*-initiated replisome progression and (ii) increased arrest at Ter sites flanking the terminus region which are bound by the Tus protein (52, 53). The occurrence of cSDR in *rnhA* mutants has been established through both biochemical and genetic assays (48). The protein DksA, which participates in avoidance or resolution of transcription-replication conflicts (54, 55), is also required for viability of *rnhA dnaA* mutants (56).

In recent work, Drolet's group showed by biochemical assays that cSDR occurs in Topo I-deficient cells (28). Ogawa and coworkers and Kaguni and coworkers also showed that specificity for replication initiation from *oriC in vitro* requires both RNase HI and Topo I (57, 58).

In this study, we examined several *topA* insertion and deletion alleles for both their viability and their ability to rescue Δ*dnaA* lethality in *E. coli*. Our results indicate that

topA-null alleles are lethal in the wild-type strain MG1655 but that they are viable in MDS42, which is an engineered derivative lacking all prophages and transposable elements (59). The null mutants of MG1655 were viable with *rpoB*35*, which encodes an RNAP variant that has been reported to alleviate the deleterious effects of transcription-replication conflicts (40, 52, 60–65). Both in MDS42 and with *rpoB*35*, the viable Topo I-deficient derivatives continued to exhibit increased negative supercoiling. One *topA* allele could also rescue Δ*dnaA* lethality, providing genetic confirmation of cSDR in Topo I-deficient strains. We propose that bacterial lethality in the absence of Topo I is caused by RNAP backtracking during transcription elongation and associated R-loop formation, which in turn lead to transcription-replication conflicts and to cSDR.

RESULTS

Description of *topA* insertion and deletion mutations and the assay to test for their viability. Three pairs of *topA* mutations were constructed on the *E. coli* chromosome by the recombineering approach of Datsenko and Wanner (66), each pair being composed of an FRT (FLP recombination target)-flanked Kan^r element and the corresponding derivative with Flp recombinase-mediated site-specific excision of the Kan^r element to leave behind a "scar" of 27 in-frame codons; these are designated below by the suffixes "::Kan" and "::FRT," respectively.

The three pairs of mutations represent the following (Fig. 1A): (i) deletion of all but the first codon and the last six codons (860 to 865) of the *topA* open reading frame (Δ*topA*), that is, similar to the various gene knockouts of the Keio collection (67); (ii) insertion beyond codon 480 in *topA* (*topA*-Ins480), this position being chosen because an earlier study had shown that a Tet^r insertion allele at this site was viable and associated with increased frequency of transposon precise excisions (11); and (iii) deletion from codon 480 to codon 860 in *topA* (*topA*-480Δ).

All the *topA* mutations were constructed and maintained in derivatives that were also Δ*lacZ* on the chromosome and carried a shelter plasmid derivative of a single-copy IncW replicon encoding trimethoprim (Tp) resistance (68) with *topA*$^+$ and *lacZ*$^+$ genes. Since this plasmid's segregation into daughter cells during cell division is not tightly controlled, around 10% of cells in a population are plasmid free. Only provided that these latter cells are viable, they grow as white colonies on Tp-free medium supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), whereas the plasmid-bearing cells grow as blue colonies on these plates. The appearance of white colonies which can be subsequently purified, therefore, is a demonstration of viability in the absence of the *topA*$^+$ shelter plasmid, and we employed similar blue-white screening assays earlier for tests of viability with other essential genes, such as *rho*, *nusG*, *dnaA*, and *rne* (69–71).

Viability of *topA* mutations in MDS42 and MG1655 *rpoB*35*. With the blue-white assay described above, we found that none of the six *topA* alleles is viable in MG1655 (Fig. 1B). These observations are consistent with those of Stockum et al. (13), who also employed a similar approach to conclude that *topA* is essential in *E. coli*.

By the same blue-white assay, we could show that all the *topA* mutations are viable in strain MDS42 and in *rpoB*35* derivatives of both MG1655 and MDS42, on both LB and defined media (Fig. 1B; also, see Fig. S1A in the supplemental material); the growth of white colonies of the MDS42 *topA* derivatives was improved in the presence of *rpoB*35*, on both media (Fig. S1B). MDS42 is a derivative of MG1655 with 14% of its genome (comprising all prophages and transposable elements) deleted (59), while *rpoB*35* is a mutation in RNAP that has been reported to render the enzyme less prone to backtracking or arrest and more accommodative of conflicts with replication (40, 52, 60–65).

In microscopy experiments (Fig. S2), cell size and morphology were unchanged with the *rpoB*35* mutation alone in both MG1655 and MDS42. Cells of the MDS42 *topA* mutant were filamented, and the filamentation was to a large extent suppressed in the *topA rpoB*35* derivative. The *topA rpoB*35* derivative of MG1655 was also moderately filamented.

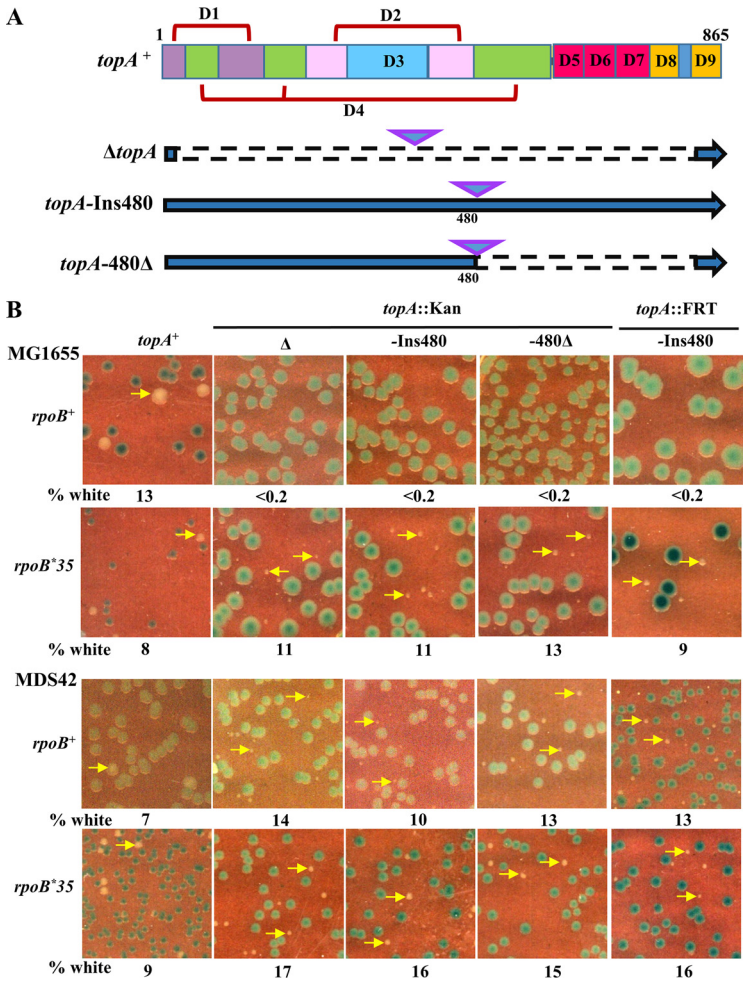

FIG 1 (A) Representations of *topA*$^+$ ORF delineating the encoded protein's domains D1 to D9 (adapted from references 87 and 88) and of the constructed *topA* alleles (three pairs) wherein the interrupted line segments represent deletions and each inverted triangle represents the pair comprising either Kan^r insertion (::Kan allele) or its FRT derivative (::FRT allele). Domains D1, D2, and D4 of Topo I are each composed of two or more polypeptide segments that are split in the linear representation but are in proximity to one another in the three-dimensional protein structure. (B) Blue-white screening assay, on LB medium, of MG1655 and MDS42 strain derivatives with the *topA*$^+$ shelter plasmid pHYD2390 and the different *topA* alleles as indicated at the top of each column; the *rpoB* allele status is indicated to the left of each row. Examples of white colonies are marked by yellow arrows. From left to right, strains used for the panels were pHYD2390 derivatives, as follows: row 1, GJ13519, GJ15603, GJ15604, GJ15688, and GJ16921; row 2, GJ16703, GJ16813, GJ16814, GJ16815, and GJ16854; row 3, GJ12134, GJ16816, GJ16817, GJ16818, and GJ18977; and row 4, GJ16819, GJ16820, GJ16821, GJ16822, and GJ17777.

Growth rate experiments in liquid cultures did not yield reliable data because of extended lag times and accumulation of suppressors in the *topA* derivatives. Suppressor accumulation has also been documented earlier for *topA* mutants by other workers (8, 13). Based on the observation that the "white" *topA* mutant clones in the blue-white screening assay grow to colonies of around 10^8 cells in 48 h, we estimated a doubling time of around 100 min.

For reasons that are explained in Discussion, we tested whether suppression of *topA* lethality by *rpoB*35* in MG1655 is abolished in the absence of the UvrD DNA helicase in the cells. In the blue-white assay, viable colonies of MG1655 *rpoB*35 topA* were obtained even in a Δ*uvrD* background, indicating that the suppression is UvrD independent (Fig. S3A).

Rescue of *topA* lethality in MDS42 or by *rpoB*35* is not due to reversal of hypernegative supercoiling. Lethality caused by loss of Topo I is associated with greatly elevated levels of negative supercoiling *in vivo*, and at least some suppressors

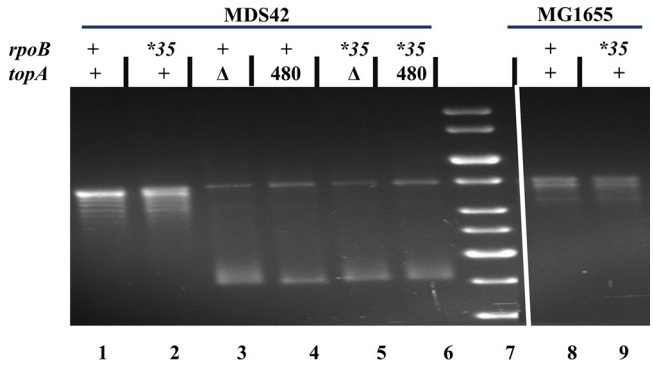

FIG 2 Supercoiling status of reporter plasmid pACYC184 in *topA*$^+$ and *topA* derivatives, as determined by chloroquine-agarose gel electrophoresis. Genotypes at *topA* and *rpoB* loci are indicated on top of each lane; for *topA*, Δ and 480 refer to Δ*topA*::FRT and *topA*-Ins480::FRT, respectively. Strains were pACYC184 derivatives: lane 1, GJ12134; lane 2, GJ16819; lane 3, 18976; lane 4, GJ18977; lane 5, GJ17776; lane 6, GJ17777; lane 8, GJ18601; lane 9, GJ18910. Lane 7, DNA size standards.

of inviability, such as mutations in *gyrA* or *gyrB* and overexpression or amplification of genes encoding topoisomerases III or IV, also confer reversal of the hypernegative supercoiling phenotype (14–16, 18, 19; for reviews, see references 4 and 24). To examine whether the viability of *topA* null mutants in MDS42 and with *rpoB*35* is correlated with reversal of hypernegative supercoiling, we determined their supercoiling status, by chloroquine-agarose gel electrophoresis (21, 72) of a reporter plasmid pACYC184 (73) in preparations made from the different strain derivatives.

The results indicate that (i) in MDS42, both *topA* mutations tested confer increased supercoiling (Fig. 2, compare lanes 3 to 6 with lanes 1 to 2); (ii) *rpoB*35* does not alter supercoiling, in both the *topA*$^+$ (compare lanes 1 and 2, or compare lanes 8 and 9) and *topA* derivatives (compare lanes 3 and 4, or compare lanes 5 and 6); and (iii) supercoiling levels are not different between the strain backgrounds of MG1655 and MDS42 for both *rpoB*$^+$ (compare lanes 1 and 8) and the *rpoB*35* mutant (compare lanes 2 and 9). We conclude that when lethality conferred by loss of Topo I is suppressed either by genome size reduction in MDS42 or *rpoB*35* or by both perturbations together, there is no concomitant reduction in the hypernegative supercoiling status of DNA in these mutants.

***topA* lethality in MG1655 is not rescued by ectopic expression of the R-loop helicase UvsW.** Ectopic expression of the phage T4-encoded R-loop helicase UvsW (74) was previously shown to rescue lethality associated with increased R-loop prevalence in several different *E. coli* mutants. The latter include strains with combined deficiency of RNases HI and HII (69) or of RNase HI and RecG (75), as well as those with deletions of genes *rho* or *nusG* involved in factor-dependent transcription termination (69).

Since Topo I deficiency phenotypes are also associated with increased occurrence of intracellular R-loops and are partially suppressed by RNase HI overexpression (18, 21–23, 26–28), we employed the blue-white assays to examine whether UvsW expression (from a P_{tac}-UvsW chromosomal construct, induced with IPTG [isopropyl-β-D-thiogalactopyranoside]) could rescue MG1655 *topA* lethality; an MG1655 Δ*rho* derivative (whose lethality is known to be rescued by UvsW) was chosen as a control. The results indicate that UvsW expression does not confer viability to the MG1655 *topA* derivative, whereas it could do so to the Δ*rho* mutant (Fig. S3B). UvsW expression was associated with impaired growth of the *topA*$^+$ blue colonies; this growth impairment was exemplified both by a marked decrease in plating efficiency and by occurrence of blue haloes around the colonies, suggestive of cell lysis. That UvsW expression is toxic to wild-type *E. coli* has been reported earlier (69).

Rescue of Δ*dnaA* lethality by Topo I deficiency. As mentioned above, Topo I deficiency was earlier shown by a biochemical assay to confer cSDR, but whether it can rescue lethality associated with loss of DnaA function (that is, the genetic assay for cSDR) has not been determined. We adapted the blue-white assay to test whether any of the

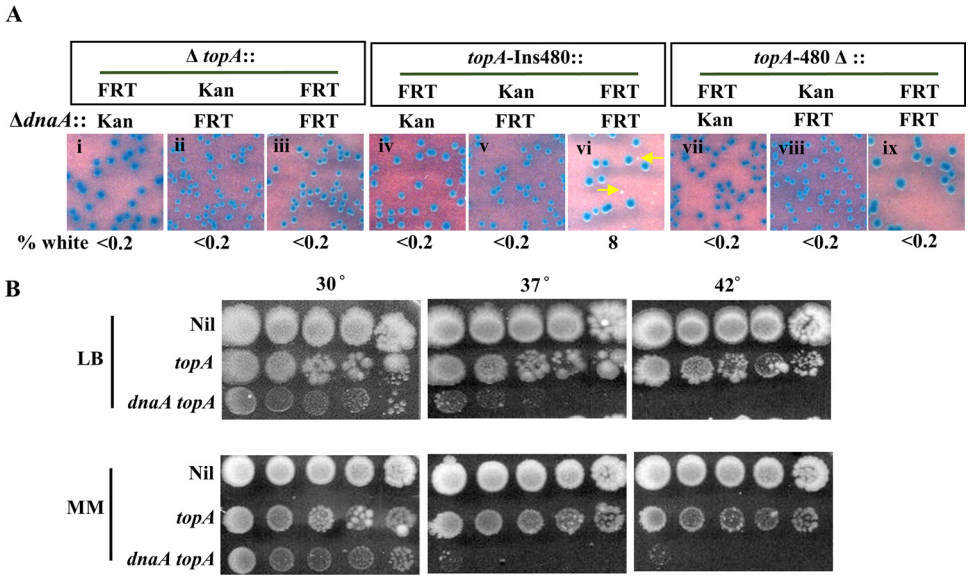

FIG 3 Suppression of Δ*dnaA* lethality by *topA*. (A) Blue-white screening assay at 30°C on glucose-minimal medium A with *topA*$^+$ *dnaA*$^+$ shelter plasmid pHYD2390 in MG1655 Δ*dnaA* Δ*tus rpoB*35* derivatives carrying different *topA* alleles; the nature of the Δ*dnaA* allele (::Kan or ::FRT) and the *topA* allele is shown at the top of each panel. Examples of white colonies are marked by arrows. Strains employed for the different panels were pHYD2390 derivatives: i, GJ17786; ii, GJ17790; iii, GJ18940; iv, GJ17787; v, GJ17791; vi, GJ18941; vii, GJ17788; viii, GJ17792; and ix, GJ18942. (B) Serial dilution spotting on LB and glucose-minimal medium A (MM) at the indicated temperatures of isogenic *topA dnaA* derivatives of MG1655 Δ*tus rpoB*35*, as follows: Nil, *topA*$^+$ *dnaA*$^+$ (GJ17784/pHYD2390); *topA*, *topA*-Ins480::FRT *dnaA*$^+$ (GJ17784); and *topA dnaA*, *topA*-Ins480::FRT Δ*dnaA*::FRT (that is, the white colony from panel vi of Fig. 4A [GJ18941]); note that the *topA*$^+$ Δ*dnaA* derivative of this strain is inviable.

topA mutations can rescue lethality associated with loss of DnaA function, by constructing a Tp^r *lacZ*$^+$ shelter plasmid that carried both *topA*$^+$ and *dnaA*$^+$. Three different *dnaA* alleles were used in these experiments: Δ*dnaA*::Kan (70), which is a Keio-style insertion-deletion that has all but the first codon and the last six codons of the 468-codon-long *dnaA* ORF removed; its FRT derivative, Δ*dnaA*::FRT (70); and *dnaA*177 (76), whose DNA sequence determination revealed that it carries both a missense mutation in codon 267 (resulting in a Thr-to-Ilv substitution) and an amber nonsense mutation in codon 450. The strains also carried Δ*tus* and *rpoB*35* mutations, which facilitate cSDR-directed chromosome duplication by overcoming the problems posed, respectively, by the Ter sites and by excessive head-on transcription-replication conflicts (52, 70, 77, 78).

Of the six *topA* mutations tested that had been shown above to be lethal in MG1655, one (*topA*-Ins480::FRT) was able to rescue lethality of Δ*dnaA*::FRT and of *dnaA*177 at 30°C on both minimal and LB media (Fig. 3 and Fig. S4A), while the others yielded no viable white colonies (Fig. 3A). Even with *topA*-Ins480::FRT, there was no rescue imposed by DnaA deficiency at 37°C or 42°C (Fig. 3B; see also row 5 in each of the panels of Fig. S5), nor were viable colonies recovered with the Δ*dnaA*::Kan allele (Fig. 3A). On the other hand, Δ*dnaA*::Kan lethality was rescued by other cSDR-provoking mutations such as *rnhA* and *dam* (data not shown).

Two distinct and interesting interpretations are suggested from these data: (i) unlike the other five *topA* alleles, *topA*-Ins480::FRT might possess a low level of DNA relaxation activity (since it encodes a full-length polypeptide with just a 27-amino-acid linker inserted between residues 480 and 481 of Topo I) which is not sufficient for viability *per se* in MG1655 but nonetheless is necessary for viability in the derivatives whose sole source of chromosome duplication is cSDR and (ii) expression of the essential *dnaN* gene immediately downstream of, and in the same operon as, *dnaA* is achieved from a fortuitous outward reading promoter in the Kan^r element of the

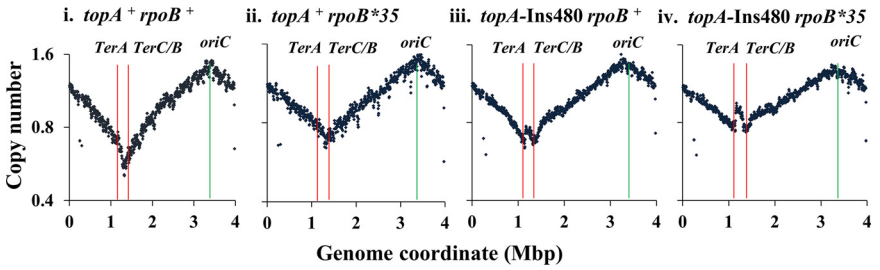

FIG 4 Copy number analysis by deep sequencing in *topA* mutant derivatives of MDS42. Relative copy numbers are plotted as semi-log graphs for overlapping 10-kb intervals across the genome (the relevant genotype of each strain is indicated at the top of each panel); positions of *oriC*, TerA, and TerC/B are marked, and the gap at around 0.3 Mbp in each of the plots corresponds to the *argF-lac* deletion present in the strains. Strains used for the different panels are as follows: i, GJ12134; ii, GJ16819; iii, GJ18977; and iv, GJ17777.

Δ*dnaA*::Kan allele, but this promoter is rendered inactive under Topo I-deficient conditions.

Notwithstanding these unusual features, our data clearly establish that cSDR in a Topo I-deficient derivative can act to rescue the lethality associated with total absence of DnaA in the cells. This viability is contingent on absence of the Tus protein (Fig. S4B). On the other hand, the DinG helicase, which has been shown to be needed for Δ*dnaA* viability of RecG- or Dam-deficient cells (70), was not required in the Topo I-deficient strain, nor did its absence impede viability of the *topA* mutant in the *dnaA*$^+$ derivative (Fig. S4C).

Copy number analysis of different chromosomal regions in *topA* mutants. In an exponentially growing population of bacterial cells, DnaA-initiated replication imposes a bidirectional gradient of copy number for different regions of the circular chromosome, with the peak near *oriC* and trough in the terminus region (53). If a *dnaA*$^+$ strain also suffers a perturbation that activates cSDR (such as deficiency of RNase HI, RecG, Dam, or multiple DNA exonucleases), the DNA copy number pattern is characterized by superposition of a "midterminus peak" on the bidirectional gradient described above (52, 70, 77–82). Previously, we proposed that the midterminus peak represents a population aggregate of replication forks progressing from stochastically firing cSDR origins that are widely distributed across the genome (53, 70), although other groups have suggested that it represents a discrete origin of replication (52, 77–79), or occurrence of overreplication when oppositely directed forks converge at the terminus (80, 81).

Brochu and coworkers have shown earlier that Topo I-deficient mutants exhibit the midterminus peak (19), but their strains also carried additional genetic changes, such as a *gyrB*(Ts) mutation and amplification of the genes encoding subunits of topoisomerase IV. For our DNA copy number analysis studies, we used *dnaA*$^+$ strains of the MDS42 background without or with *rpoB*35* and additionally with the *topA*-Ins480::FRT mutation (that is, the allele associated with rescue of Δ*dnaA* inviability).

The whole-genome sequence reads obtained from each of the strains were aligned to the MDS42 reference sequence, and normalized read counts for the different chromosomal regions were determined. No suppressor mutation in any of the candidate genes was identified in the *topA* mutants, while the presence of the *topA* mutation itself and of the CAC-to-CAA codon change (His-to-Gln substitution) associated with the *rpoB*35* allele (60) was confirmed in each of the relevant strains.

The parental (*topA*$^+$ rpoB$^+$) strain exhibited the expected bidirectional copy number gradient from *oriC* to Ter (Fig. 4, panel i), which was also largely preserved in its *rpoB*35* derivative (Fig. 4, panel ii). The *topA* mutant derivatives of both these strains showed distinct midterminus peaks superimposed on the *oriC*-to-Ter gradient (Fig. 4, panels iii and iv, respectively).

These results therefore confirm that a *topA* mutation capable of conferring Δ*dnaA* viability is associated with a midterminus peak of DNA copy number in *dnaA*$^+$ derivatives.

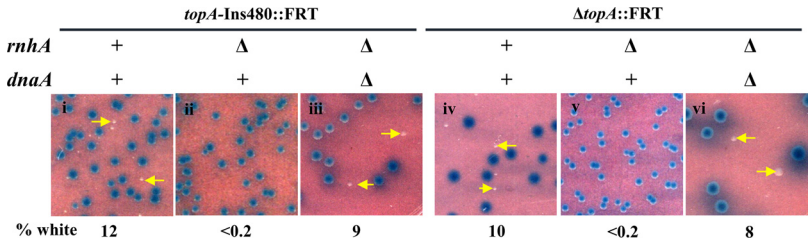

FIG 5 Synthetic *topA rnhA* lethality, suppressed by Δ*dnaA*. Blue-white screening assay at 30°C on glucose minimal medium A with the *topA*$^+$ *dnaA*$^+$ shelter plasmid pHYD2390 in MG1655 Δ*tus rpoB*35* derivatives carrying different alleles of *topA*, *rnhA*, and *dnaA* as indicated at the top of each panel. Strains employed for the different panels were pHYD2390 derivatives: i, GJ17784; ii, GJ19609; iii, GJ18951; iv, GJ17783; v, GJ19608; and vi, GJ18983.

Mutual suppression between lethal mutations: loss of DnaA suppresses *topA-rnhA* synthetic lethality. Deficiency of either Topo I or RNase HI is associated with cSDR, and Stockum et al. (13) as well as Drolet and coworkers (26, 29) previously reported lethality or aggravated sickness in the double-deficient strains. We too found in this study that introduction of the *rnhA* mutation into otherwise viable *topA* derivatives (that is, in the MG1655-derived strain with *rpoB*35* and Δ*tus* mutations) confers synthetic lethality; the two *topA* alleles tested were the FRT derivatives of *topA*-Ins480 and Δ*topA* (Fig. 5, compare panels i and ii for the former and iv and v for the latter). The lethalities were rescued in the presence of Δ*dnaA* (Fig. 5, panels iii and vi, respectively), indicating that two otherwise lethal mutant combinations (*topA rnhA* and *dnaA*) could mutually suppress one another. Robust viability of the triple mutants was observed on both rich and defined media at 30°C and 37°C but less so at 42°C (Fig. S5).

We performed PCR experiments to confirm that the chromosomal *topA* locus in each of the viable triple-mutant *topA rnhA dnaA* derivatives was indeed disrupted (and had not, for example, become *topA*$^+$ by gene conversion from the wild-type allele on the shelter plasmid). Two primer pairs were used simultaneously to distinguish between the *topA*$^+$, Δ*topA*::FRT, and *topA*-Ins480::FRT alleles, which yielded amplicons of 500, 328, and 581 bp, respectively (Fig. S6A and B). The results established that the signatures for the *topA*-Ins480 and Δ*topA* mutations were present, and that the one for *topA*$^+$ was absent, in the triple-mutant strains (Fig. S6B, lanes 5 and 7, respectively).

As discussed below, these results suggest that it is excessive chromosomal replication which kills cells doubly defective for Topo I and RNase HI.

DISCUSSION

The enzymes Topo I and DNA gyrase act to maintain the homeostatic balance of DNA negative supercoiling in *E. coli*. Topo I relaxes negative supercoils, especially those occurring behind RNAP during transcription elongation, and thus prevents the nascent transcript from reannealing with the template DNA strand to form R-loops.

In this study, we confirmed that Topo I-deficient *E. coli* mutants are inviable and furthermore have identified two novel means by which the lethality can be independently and additively suppressed: (i) by deletion of the nonessential 14% of the genome comprising prophages and transposable elements, as in strain MDS42, and (ii) by the *rpoB*35* mutation, encoding an altered RNAP, which has been variously described (not mutually exclusive) to mimic the transcriptional effects of ppGpp (60), to reduce RNAP backtracking (40), and to mitigate the effects of transcription-replication conflicts by destabilizing the TEC (40, 52, 62, 63, 65). Neither of the suppressors acts by reversing hypernegative supercoiling in the *topA* mutants. We have also shown that Topo I deficiency, in the presence of additional *rpoB*35* and Δ*tus* mutations, can rescue Δ*dnaA* lethality, thereby providing genetic confirmation for occurrence of cSDR in the Topo I-deficient derivatives.

***rpoB*35* and RNAP backtracking.** As mentioned above, several workers have provided evidence that the *rpoB*35*-encoded substitution in RNAP destabilizes the TEC *in*

vitro (40, 62) and protects against transcription-replication conflicts *in vivo* (65), including during cSDR (52, 70, 77, 78). Trautinger and Lloyd (61) reported that *rpoB*35* suppresses the Ts phenotype of *greA greB* double mutants and the UV sensitivity of an *mfd* mutant, which they interpret as evidence that it may function by preventing backtracking, thus facilitating dissociation of stalled TECs. Likewise, *rpoB*35* also suppresses *rep uvrD* lethality, which has been ascribed to direct reduction of replicative barriers posed by TECs under these conditions (63).

On the other hand, there is one report from the Nudler group that RpoB*35-substituted RNAP exhibits increased backtracking *in vitro* in the presence of UvrD (64). This property of RpoB*35-RNAP appears to be strictly UvrD dependent, and the same group has shown in other studies (40) that relative to wild-type RNAP, the RpoB*35 enzyme is resistant to pausing and backtracking.

It is therefore reasonable to conclude that the RpoB*35 enzyme is in general more resistant than wild-type RNAP to arrest and backtracking during transcription elongation, except perhaps in the specific context when a high concentration of UvrD dimers occurs following DNA damage. Our finding in this study, that the suppression of *topA* lethality by *rpoB*35* is UvrD independent (Fig. S3A), is noteworthy in this context.

Mechanism of lethality in Topo I-deficient strains. The fact that *rpoB*35* restores growth to MG1655 in the absence of Topo I without affecting the hypernegative supercoiling status of the mutants suggests that it is the downstream consequences of increased negative supercoiling, namely, RNAP backtracking and impairment of TEC progression leading to transcription-replication conflicts, which are responsible for *topA* lethality. Pathological R-loop formation is also expected to be an important feature at the arrested TECs, but whether it precedes or follows RNAP backtracking remains to be determined. In the *topA* mutant, *rpoB*35* would also relieve the sickness during cSDR engendered by transcription-replication conflicts especially at the *rrn* operons.

To explain *topA* viability in MDS42, we propose that the regions of the genome that are deleted in this strain (comprising prophages and transposable elements) are preferentially enriched for sites of R-loop formation, TEC arrest and transcription-replication conflict. Loss of the proteins Rho and NusG, which are involved in factor-dependent transcription termination and reportedly in R-loop avoidance (31, 69, 83–85), is also better tolerated in MDS42 than in MG1655, and especially so in the presence of *rpoB*35* (65, 69, 86).

Finally, why does ectopic expression of the R-loop helicase UvsW not rescue *topA* lethality, although it can rescue the lethalities associated with loss of RNase H enzymes, RecG, Rho, or NusG (69, 75)? One possibility is that R-loop formation in Topo I-deficient strains is a consequence, and not a cause, of RNAP backtracking and arrest, so that R-loop removal *per se* would not mitigate the primary problem. An alternative possibility is that Topo I itself is required to relax the negative supercoils arising from UvsW's helicase action on R-loops, and hence that UvsW is unable to act efficiently to unwind R-loops specifically in the *topA* mutants. The fact that RNase HI overexpression can suppress *topA* sickness phenotypes (26, 27) lends support to the second model.

Topo I deficiency and cSDR. Martel and coworkers provided biochemical evidence for cSDR in Topo I-deficient cultures (28), which is presumably initiated from R-loops in these cells; our data establish that such cSDR is sufficient to sustain viability in the absence of DnaA, in derivatives carrying Δ*tus* and *rpoB*35* mutations. The latter two mutations are expected to facilitate the completion of replication of the circular chromosome by forks initiated from a site(s) other than *oriC* (52, 70, 77, 78). Our data also support the earlier suggestion (70) that incubation at 30°C is more permissive than that at 37°C or 42°C for rescue by cSDR of Δ*dnaA* lethality.

Of the six different *topA* mutations that were inviable in MG1655-derived strains, it was only the *topA*-Ins480::FRT allele that could confer viability to the Δ*dnaA* derivatives. As explained above, this mutation generates a modified version of Topo I in which a 27-amino-acid linker is inserted between residues 480 and 481 of the polypeptide. From the Topo I monomer crystal structure (87, 88), it is expected that the linker

is situated at or near the junction between residues that make up domain D2 and those that make up domain D4; it is therefore possible that the linker allows the (albeit inefficient) folding of the polypeptide to yield a correct tertiary structure. The residual Topo I activity of this protein might be needed for proper chromosome segregation after cSDR in the Δ*dnaA* mutants (20).

***oriC*-initiated replication contributes to *topA-rnhA* synthetic lethality.** We have shown that although the Δ*topA* and Δ*rnhA* combination is synthetically lethal, the Δ*topA* Δ*rnhA* Δ*dnaA* mutant is viable. Thus, *oriC*-initiated replication is a contributor to *topA rnhA* toxicity, which suggests that it is excessive replication (sum of that from *oriC* and cSDR, the latter contributed additively by both *rnhA* and *topA*) which confers toxicity. These results are in agreement with those from Usongo and coworkers (20, 29), who reported earlier that mutations affecting either replication from *oriC* or replication restart functions can alleviate the sickness of cells deficient for both Topo I and RNase HI activities.

MATERIALS AND METHODS

Growth media, bacterial strains, and plasmids. The rich and defined growth media were, respectively, LB and minimal A with 0.2% glucose (89), and unless otherwise indicated, the growth temperature was 37°C. Supplementation with X-Gal and the antibiotics ampicillin, kanamycin (Kan), tetracycline (Tet), chloramphenicol (Cm), and trimethoprim (Tp) were at the concentrations described earlier (90). Isopropyl-β-D-thiogalactoside (IPTG) was added at the indicated concentrations. *E. coli* strains used are listed in Table S1 in the supplemental material.

Plasmids described earlier include pMU575 (Tp^r, single-copy-number vector with *lacZ*$^+$) (68); pACYC184 (Tet^r Cm^r, p15A replicon) (73); pHYD2388 (70) and pHYD2411 (69) (Tp^r, pMU575 derivatives with, respectively, *dnaA*$^+$ and *rho*$^+$); and pKD13, pKD46 and pCP20, described by Datsenko and Wanner (66), for recombineering experiments and Flp recombinase-catalyzed excision between a pair of FRT sites. The construction of two derivatives of plasmid pMU575 is described in the supplemental material: pHYD2382, carrying *topA*$^+$, and pHYD2390, carrying *topA*$^+$ *dnaA*$^+$.

Blue-white screening assays. To determine lethality or viability of strains with chromosomal *topA* or *dnaA* mutations, derivatives carrying the shelter plasmid pHYD2382 (*topA*$^+$) or pHYD2390 (*topA*$^+$ *dnaA*$^+$) were grown overnight in Tp-supplemented medium, subcultured into medium without Tp for growth to mid-exponential phase, and plated at suitable dilutions on X-Gal plates without Tp. The percent white colonies was determined (minimum of 500 colonies counted), and representative images were captured.

Plasmid supercoiling assays. Strains carrying plasmid pACYC184 were grown in LB to mid-exponential phase, and plasmid preparations were made with the aid of a commercial kit. Plasmid supercoiling status in each of the preparations was determined essentially as described elsewhere (72), following electrophoresis on 1% agarose gels with 5 μg/ml chloroquine at 3 V/cm for 17 h.

Copy number analysis by deep sequencing. Copy number determinations of the various genomic regions were performed essentially as described (70). Genomic DNA was extracted by the phenol-chloroform method from cultures grown in LB to mid-exponential phase, and paired- and single-end deep sequencing was performed on Illumina platforms to achieve around 100-fold coverage for each preparation. Sequence reads were aligned to the MDS42 reference genome (accession number AP012306.1), and copy numbers were then determined by a moving-average method after normalization of the base read count for each region to the aggregate of aligned base read counts for that culture.

Other methods. Procedures were as described for P1 transduction (91) and for recombinant DNA manipulations, PCR, and transformation (92). Different chromosomal *topA* mutations were generated by recombineering (66) as described in the supplemental material. For microscopy experiments, cells from cultures grown in LB to mid-exponential phase were immobilized on 1% agarose pads and visualized by differential interference contrast imaging with the aid of a Zeiss Axio Imager Z2 microscope.

Data availability. Genome sequence data from this work have been submitted under accession number PRJNA670792 and are available for public access at https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA670792.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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We declare that there are no conflicts of interest.

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