The Role of DNA Topoisomerases in the Completion of DNA Replication

by

Parisa Mokhtari

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Thesis Committee:
Justin Courcelle, Chair
Anne Thompson
Michael Bartlett

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Abstract

Accurate replication of the genome is essential to maintain cellular life. Completing DNA replication on the chromosome is central to this task and involves several enzymes, including RecBCD, RecG and SbcCD-ExoI, which process, join, and resolve the two convergent replication forks. This event is certain to generate a number of structural complexities involving supercoiling, tangles and knots as the two DNA replication forks converge which must be resolved to maintain the integrity of the genome. Structural knots and tangles generated during DNA replication, transcription and segregation are typically resolved by DNA topoisomerases. *E. coli* encodes four DNA topoisomerases—two type I (Topo I and Topo III) and two type II (Topo IV and DNA gyrase). Here, we used mini-chromosomes that require replisomes to converge to identify which topoisomerases participate in processing substrates during completion. I show that mutants lacking Topo I, but not Topo III, are impaired in their ability to maintain substrates containing convergent replication forks. Additionally Topo IV, but not gyrase, contributes to the completion step on plasmids. I then confirmed these results on the *E. coli* chromosome where distinct phenotypes were observed in each case. Impaired Topo I function led to amplifications where forks converge, whereas loss of Topo IV activity resulted in extensive degradation and loss of this chromosome region. I discuss models in which Topo I plays a general role in maintaining supercoiling to allow replisomes to converge, whereas Topo IV plays a more specific role and is required to allow the nascent strand ends of convergent forks to be joined.

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Chapter 1

Introduction

Current model of completing DNA replication

Accurate replication of the genome is central to maintaining cellular life. The process can be divided into three stages: replication initiation, elongation and completion. Each of these steps is highly regulated to ensure that both daughter cells inherit an identical copy of the genetic information. Whereas initiation and elongation have been well characterized (reviewed in 1-3), less is known about how replication is completed. At a minimum, the steps of completion must involve fork convergence, joining of the nascent DNA strands, replisome disassembly and decatenation of the completed chromosomes (reviewed in 4; 5). To accurately complete replication, cells utilize a multi-step pathway that is capable of recognizing sequence pairs and limiting convergent replication forks to their precise point where sequences have doubled (6-8). Failure to accurately join and complete any single event where replication forks converge would result in mutations. This can occur through the insertion of an incorrect base, duplications, deletions or rearrangements if the nascent DNA ends are joined at the wrong time or place, or cell death if the convergent strands fail to join. Despite its complexity, the reaction must be highly efficient, as it occurs thousands of times per division in human cells.

Completion has been challenging to study in human cells, in part because origins are poorly defined and utilized with varying efficiency, frequency and timing, making these loci highly variable between cells and cell cycles (9-11). In contrast, *E. coli* has served as a useful model to begin to dissect how this fundamental process occurs, as forks converge within a single defined 400 kb region on the genome, located opposite to its bidirectional

origin of replication (12). These events are contained within this region by multiple termination (*ter*) sequences, which bind Tus, a protein that limits replication forks from progressing outside this region (13). Although Tus confines completion to this region, it does not appear to directly participate in the reaction since mutants lacking Tus or *ters* complete replication normally (14, 15).

Although *tus* mutants are aphenotypic, several gene products have been identified that impair the completion reaction when mutated and result in abnormalities specifically at loci where replication forks converge (6-8). Current models suggest that converging replisomes transiently bypass each other, creating an over-replicated region that contains a third copy of the genetic information (Figure 1.1). The over-replicated intermediate is observed *in vitro* in reconstituted systems (16) and *in vivo* on the chromosome in exonuclease mutants that allow these intermediates to persist (8, 17, 18). RecG encodes an enzyme with helicase and branch migration activities and plays a critical role in limiting the amount of over-replication that occurs where replication forks meet (6, 19). The RecG helicase appears to be important to prevent forks from re-initiating, as large amplifications arise at sites where forks converge in its absence (6, 7). One possibility is that the enzyme acts as a translocase and removes the 3' end of the nascent DNA strand, preventing DNA polymerase from extending the strand. It is thought that the intermediate structure created by this over-replication is initially processed by SbcCD and ExoI, a cohesion-like structure-specific nuclease and 3' to 5' exonuclease (18). The over-replicated substrate is incised and resected by the combined action of SbcCD and ExoI (7, 20). In *sbcCDxonA* mutants, these over-replicated regions where replication forks converge remain, leading to genomic instabilities at these loci (5, 7, 8, 20).

Following incision, the heterotrimeric RecBCD helicase-nuclease complex, processes and resects this region (7, 21, 22). In the absence of RecBCD, the nascent DNA ends persist, and extensive degradation occurs in the chromosome region where forks converge, severely compromising growth and viability (7). This degradation implies that RecBCD function is required to join nascent strand ends of convergent forks, either directly or indirectly, to complete the reaction. The enzyme also appears to play a role in recruiting, either directly or indirectly, DNA polymerase I and DNA ligase, which fill in the gaps and join the DNA strands together in order to complete replication (7). Consistent with this, inactivation of DNA ligase also leads to degradation at these loci (23). Importantly, the completion reaction does not require recombination, as *recA* mutants complete replication normally (7). However, RecA is required for many of the amplifications and duplications that arise when completion is impaired, suggesting that the recombination observed in this process is aberrant (7, 8, 24). While we have a better understanding of the different enzymes involved in the completion of replication, a lot is still unknown about how these enzymes interact with each other, in what sequence do they function and how they are regulated.

Topological challenges during DNA replication

Topological challenges in a cell arise from processes associated with both transcription and replication and involve relaxing positively or negatively supercoiled DNA (3, 25, 26). During transcription, topological challenges arise as RNA polymerase rotates around the template DNA. These topological structures include positive supercoils in front of the transcription complex and negative supercoils behind the transcription complex (27).

During replication elongation, positive supercoils accumulate in front of the forks as replication progressively unwinds the duplex DNA (reviewed in 3, 4). Precatenanes can then arise behind the fork as the replisome swivels to relieve this stress, twisting and intertwining the sister duplexes in the newly replicated region (28-30). Improper processing of these intermediates can impair duplication of the genome, impede gene expression and generate illegitimate R-loops and D-loops, which can produce instabilities on the genome (31-33).

As the completion of replication begins, the cell likely faces multiple unique topological challenges. As replisomes converge, the space to resolve these tangles diminishes and may require unique enzymes to access these regions or structures. Topological constraints such as these are typically processed by DNA topoisomerases, making it likely that these enzymes will play a role in the completion of replication. It remains unclear, at present, how they are resolved or processed.

DNA topoisomerases in *E. coli*

E. coli encodes four DNA topoisomerases which are classified as type I or type II enzymes, based on their mechanism of action. Topoisomerases form transient protein-DNA complexes that introduce a break on either one (type I) or both (type II) DNA strands, before swiveling and rejoining the DNA to relieve or introduce twisting in the double-stranded helix (reviewed in 3). Topo I and III, encoded by *topA* and *topB* respectively, are the type IA topoisomerases. Type IA topoisomerases preferentially bind and form covalent interactions with single-stranded DNA (34, 35). As seen in Figure 1.3, type IA topoisomerases use an enzyme-bridging mechanism to pass DNA through a single-strand break (36).

Topo I is essential, although null mutants can be obtained that have suppressor mutations, typically arising in other topoisomerases (31, 37-40). Hypomorphic and suppressed *topA* mutants display altered transcription profiles and reduced replication rates implying a general role in both processes (31, 37, 40-42). *In vitro*, Topo I relaxes supercoiled plasmids and is thought to relieve negative supercoiling *in vivo* (43, 44). An *in vivo* study looking at *topA* mutants found an accumulation of hypernegative supercoiling, as seen by the presence of a mid-terminus peak in the replication profile of a *topA* mutant (40). It is speculated that the downstream effects of hypernegative supercoiling, such as the halting of the transcription elongation complex, which can lead to head-on conflicts between replication and transcription machinery, is what causes lethality in *topA* mutants (40). The lethality of Topo I mutants can be rescued by the acquisition of compensatory mutations, often in genes encoding DNA gyrase or Topo IV (37). Shishido et al. analyzed knotted forms of plasmid DNA through gel electrophoresis and electron microscopy in *topA* mutants suppressed by compensatory mutations in DNA gyrase. In these mutants, there was an increase in positive supercoiling (45). Studies have shown that mutations in DNA gyrase lead to decreased enzymatic activity and increased positive supercoiling, which suppress the effects of excessive negative supercoils in *topA* mutants (37, 46). Other suppressor mutations in *topA* mutants include the upregulation of the *parEC* operon, which encode Topo IV, a type II topoisomerase (47). An *in vitro* study testing the ability of Topo IV to relax supercoiled DNA proposed that Topo IV is capable of relaxing DNA in the absence of Topo I (38). This suggests that upregulating Topo IV activity compensates for the absence of Topo I and minimizes the effects of *topA* mutations (38, 48).

Topo III is the only nonessential topoisomerase in *E. coli*. It relaxes negative supercoiled plasmid substrates *in vitro* and resolves precatenanes (49, 50). An *in vitro* study demonstrated that a precatenated DNA substrate was resolved by adding Topo III (51). In addition to precatenane resolution, Topo III has been speculated to play roles in homologous recombination (51). This was based on evidence of increased RecA-independent recombination frequency in *topB* null cells (51). Yet, the enzymatic processing or cellular events that lead to this outcome are still unknown. Other evidence suggests Topo III plays a role as a decatenase in the cell. Topo III associates with the RecQ helicase and is capable of decatenating plasmids under these conditions *in vitro* (51, 52). Based on its association with RecQ, its suppressive role in sister-chromatid exchanges, and its classification as a type I topoisomerase, it has been suggested to play a role in resolving precatenanes post-replication (50, 53, 54). *topB* null cells grow normally; however, microscopic observations indicate modest cell disorganization during nucleoid separation and exhibit elevated levels of cell filamentation (50, 51). These observations have been used to suggest Topo III may be involved in helping the segregation of fully replicated chromosomes (51). During the completion of replication, Topo III is could speculatively help to manage accumulation of positive supercoils by resolving precatenanes and is involved in the final separation of the fully replicated chromosomes, however this possibility has not yet been examined experimentally.

Type II topoisomerases include DNA gyrase and Topo IV. DNA gyrase is encoded by *gyrA* and *gyrB*; Topo IV is encoded by *parE* and *parC* (reviewed in 3). Type II topoisomerases are heterotetrameric that use ATP hydrolysis to transport a segment of DNA through a double-stranded gap (Figure 1.3) (55, 56). *In vitro*, DNA gyrase is able to relieve

positive supercoils and catalyze negative supercoils in the presence of ATP (29, 57). DNA gyrase, along with Topo I, is thought to counteract and balance or maintain supercoiling levels in the cell (58). DNA gyrase specifically relaxes positive supercoils while Topo I relaxes negative supercoils (reviewed in 3; 43, 58). The ability of DNA gyrase to remove positive supercoils ahead of the replication fork supports the progression of replication fork movement and nascent chain elongation (25). This is supported by an *in vitro* study of replication fork movement in the presence of DNA gyrase (25). As previously mentioned, there is evidence that mutations of the two DNA gyrase genes can compensate for the loss of Topo I functionality with respect to viability (37). Mutations gyrase A that decrease in the protein's activity inhibits the introduction of additional negative supercoiling (59). DNA gyrase and Topo I also affect the formation of R-loops. This was shown by determining the ability of DNA gyrase and Topo I to resolve transcription-related supercoils in an *in vitro* system (58). *In vivo*, inactivation of DNA gyrase slows the rate of DNA replication, leading to the idea that the protein travels with the replication machinery to relieve the positive supercoils generated during replication (60, 61).

Topo IV has been proposed to have many roles, including several that overlap in function with DNA gyrase. Topo IV and DNA gyrase share about 40% homology at the amino acid level between both enzymes (38). Most evidence suggests Topo IV plays a role in resolving catenated molecules. *In vitro*, the rate of decatenation by Topo IV is significantly more efficient compared to the rate of decatenation by DNA gyrase (25, 26, 29, 62). Temperature sensitive Topo IV mutants accumulate catenated plasmids at nonpermissive temperatures (26, 63). Additionally, it has been supported that catenanes derived from DNA synthesis are specifically resolved by Topo IV and not DNA gyrase *in*

vivo (63). Another function of Topo IV, one also found in DNA gyrase, is its ability to act as a replication swivel (60). This is yet another way in which the type II topoisomerases promote replication fork progression (60).

There are conflicting views about where Topo IV may act in the cell. Some evidence based on single molecule, fluorescence microscopy which labeled Topo IV and a replisome-associated protein suggest Topo IV travels with the replisome or behind it (64). However, recent genomic studies based on measuring Topo IV cleavage sites through ChIP sequencing suggest Topo IV primarily acts in the resolution at the DNA terminus (65, 66).

Research Overview

Identifying whether a specific topoisomerase participates in the completion of DNA replication is challenging because they are often essential, and broadly involved in a range of metabolic activities that elicit pleiotropic effects *in vivo*. While prior research has identified the general biochemical activities associated with the four DNA topoisomerases in *E. coli*, the specific functions of these topoisomerases during cell growth and metabolism remain unknown. Here, I focus on their role during the completion phase of DNA replication. Understanding these last steps of DNA replication will add to our understanding of how accurate duplication of the genome occurs.

To identify the DNA topoisomerases that participate in the completion of replication, I compared plasmids whose replication requires replisomes to converge with those that do not, to identify effects specific to the completion phase of replication. I used 2D gel electrophoresis to identify the structural intermediates that DNA topoisomerases act upon during the completion reaction. Finally, I confirmed these observations directly on the *E. coli* chromosome. These results suggest that Topo I and Topo IV play a role in the completion step of DNA replication.

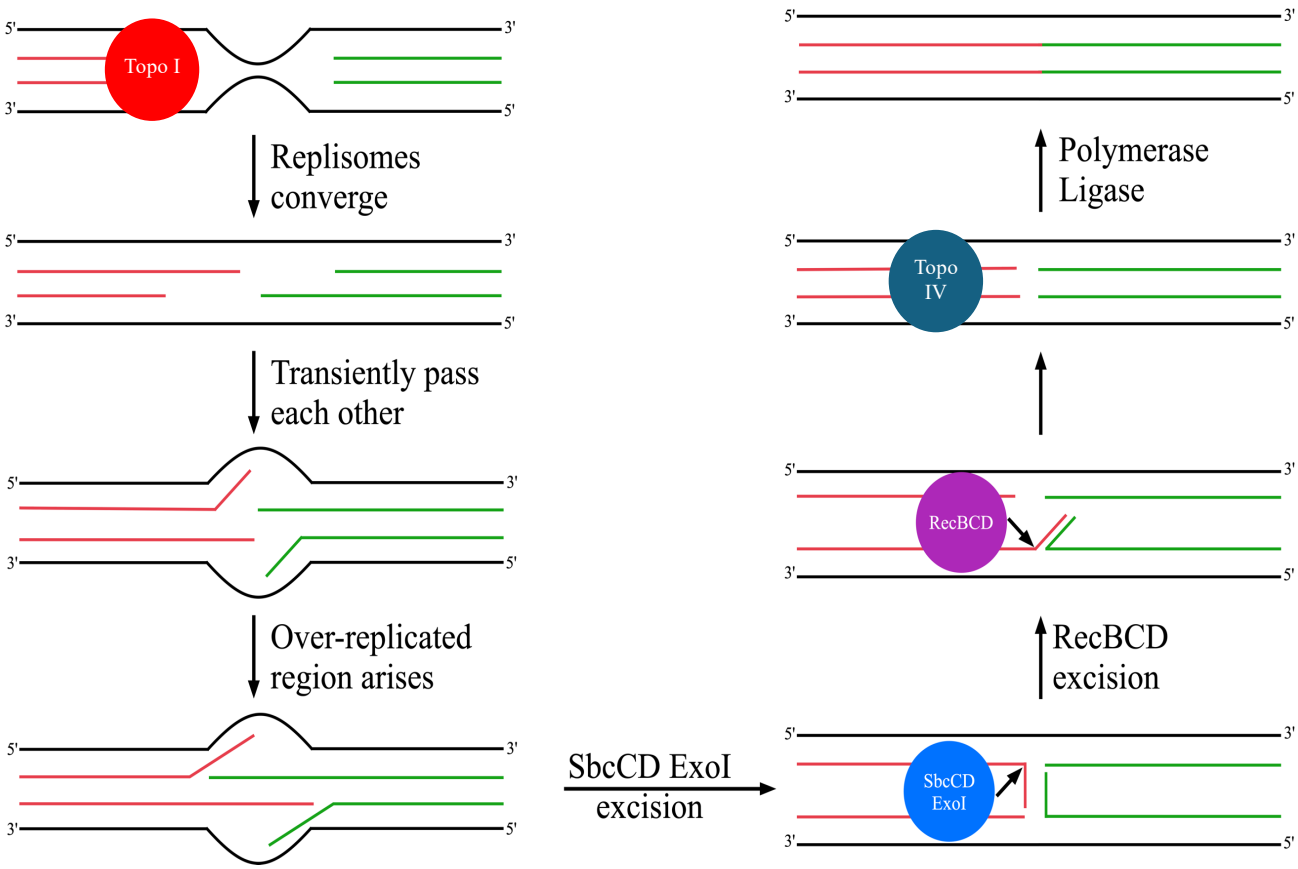

Figure 1.1. Current model of completion of replication. Replication forks converge within the terminus region of the chromosome then transiently pass each other to create an over-replicated region (8, 17, 18). It has been proposed that this overreplicated region is excised by the actions of SbcCD-ExoI and RecBCD (5, 7, 8, 20-22). It is unknown whether RecBCD directly or indirectly recruits DNA ligase to join nascent strand ends together (23).

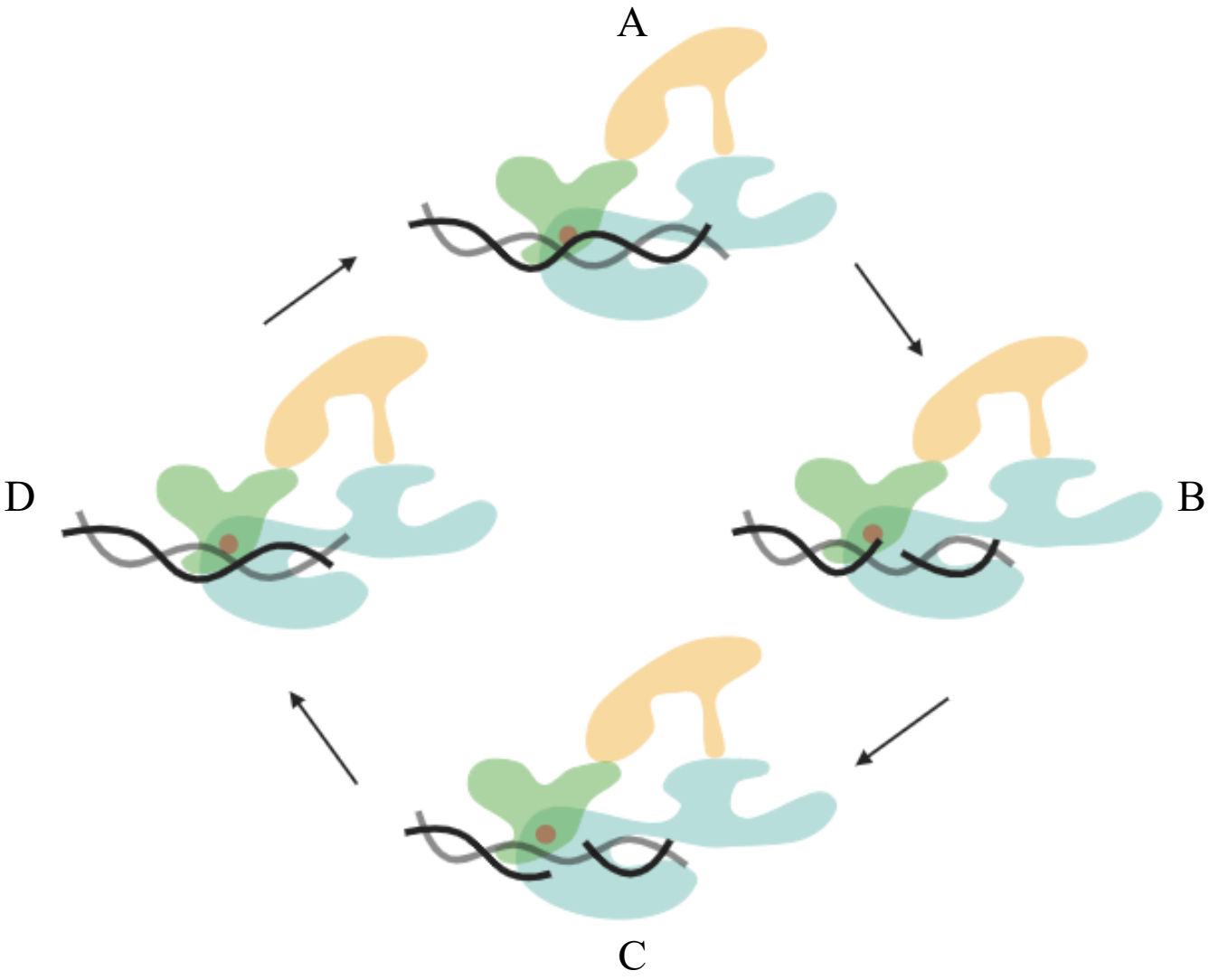

Figure 1.2. Mechanism of type I topoisomerases. Type I topoisomerases are able to reduce linking number by 1 by making a single-stranded DNA cut. In brief, the type I topoisomerase covalently binds to a DNA segment (part A) and makes a temporary cut through one of the DNA strands (part B). This leads to the formation of topoisomerase-DNA intermediate, as seen in part B. Once this intermediate is formed, one of the strands (gray strand in part C of the figure above) is able to pass through the temporary gap created by the topoisomerase (part C). Once the supercoil has been removed, the cleaved segment is religated, leading to a molecule that has a linking number decreased by 1 (part D), compared to the starting molecule (part A). The active site tyrosine, as represented by the red dot, plays an important part in this enzymatic reaction (reviewed in 67).

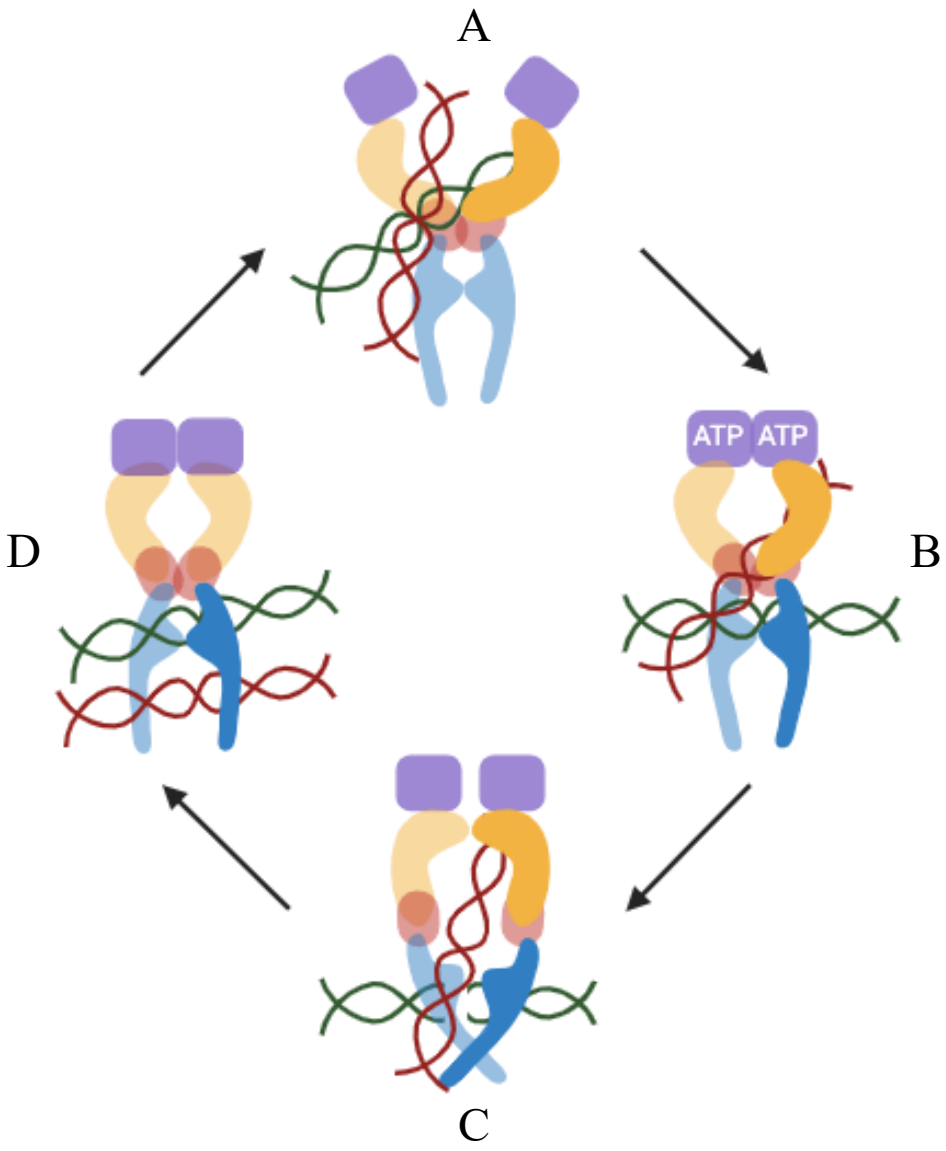

Figure 1.3. Mechanism of type II topoisomerases. Whereas type I topoisomerases are able to reduce supercoiling by a linking number of one, type II topoisomerases are able to reduce supercoiling by a linking number of two. Type II topoisomerases capture a region of the chromosome that contains intertwined duplex DNA (for example, part A in the figure above). The type II topoisomerase captures this region (part B) and makes a double-strand cut (part C). The other duplex DNA can pass through the gap created by this cut. Once the DNA is passed through, the DNA is religated and let go by the type II topoisomerase (part D). One important distinction between type I and II topoisomerases is type II topoisomerases require ATP to undergo the strand-passage mechanism (68).

Chapter 2

Materials and Methods

Strains and plasmids

The parental strain used for this study is BW25113, which has the genotype *rrnB3* Δ*lacZ*4787 *hsdR*514 Δ(*araBAD*)567 Δ(*rhaBAD*)568 *rph*-1. BW25113 *ymja*::FRT-minikan (JW1288), BW25113 *yieL*::FRT-minikan (JW5612), and BW25113 *topB*::FRT-mini-kan (JW1752) are part of the Keio single gene knockout mutant collection (69). CL4079 (BW25113 *sbcD*::FRT-mini-kan *topA2103*) containing a single base pair deletion in *topA* at nucleotide 2103 was isolated as a spontaneous secondary mutation that resulted in plasmid instability in laboratory stocks of BW25113 *sbcD*::FRT-min-kan. To construct a BW25113 *topA2103* mutant, the kanamycin-resistance cassette from the *sbcD* deletion mutant was first removed using FLP recombinase expression from the pCP20 plasmid as described previously (70, 71). *topA2103* was then linked to a kanamycin-resistance cassette approximately 25 kb away by P1 transduction of *ymjA*::Kan from JW1288 and selecting for resistance to kanamycin and plasmid instability to generate CL5110 (BW25113 *sbcD*::FRT *topA2103 ymja*::FRT-mini-kan). A second *topA* mutation, Δ(*topA-cysB*), was similarly linked to a kanamycin-resistance cassette by P1 transduction of *ymjA*::kan from JW1288 into RFM475 (31) and selecting for kanamycin resistance and cysteine auxotrophy to generate CL5427. CL5379 (large colony morphology) and CL5380 (small colony morphology) were made by P1 transduction of Δ(*topA-cysB*) *ymjA*::kan from CL5427 into BW25113. All strains were further verified by genome sequencing. CL5428 was made by P1 transduction of *parE*ts::Tn10 from MG1655 *parE*ts::Tc (72) into BW25113 then selected for loss of tetracycline resistance using chlortetracycline hydrochloride as

previously described (73) to generate CL5429. A temperature-sensitive mutation in *gyrB* was linked to a kanamycin-resistance cassette by P1 transduction of *yieL*::kan, located approximately 25 kb away from *gyrB*, from JW5612 into RFM445 (31) and selected for kanamycin resistance and temperature sensitivity to generate CL5328. CL5431 was made by P1 transduction of *gyrB*ts *yieL*::kan from CL5328 into BW25113. CL5429 (BW25113 *parE*ts) and CL5431 (BW25113 *gyrB*ts *yieL*::kan) were verified for temperature sensitivity.

Plasmid pBR322 used in this study contains a unidirectional origin of replication, which has been described previously (74) and confers ampicillin and tetracycline resistance. pCL01 plasmid used in this study contains a bidirectional origin of replication from bacteriophage λ (75) and ampicillin- and chloramphenicol-resistance cassettes.

Plasmid assay

Electro-competent cells were prepared by growing a 100-fold dilution of a fresh overnight culture in 50-mL Luria-Bertani broth without salt (LB, no NaCl) to an OD_{600} of 0.4. Strains were grown at 37°C or at 30°C for the temperature-sensitive strains. Cells were pelleted then washed with 50-mL of ice-cold 10% glycerol then resuspended in 200-300-µL of ice-cold 10% glycerol, and immediately frozen at -80°C. pBR322 and pCL01 were combined in a single mixture such that equivalent transformants per viable cells were obtained for each plasmid in wild-type cultures. The same plasmid mixture was then used for all strains analyzed. 50-µL of competent cells were mixed with the plasmid mixture, electroporated at 2.5 kV, capacitance 25 µF, resistance 200 Ohms, and allowed to recover in 1-mL SOC media for 60 minutes at 37°C. Transformation reactions were then 10-fold serially diluted and spotted in triplicate 10-µL aliquots on sets of on LB plates with no

antibiotic supplementation, 15 μg/mL tetracycline or 20 μg/mL chloramphenicol to determine the number of viable cells, pBR322, and pCL01 transformants, respectively. Plates were incubated overnight at 37°C and colonies were counted the next day. The relative transformation efficiency was calculated as the ratio of transformants per viable cells in the mutants to the transformants per viable cells in wild-type cultures.

For temperature-sensitive strains, transformation efficiencies were compared between the permissive (30°C) and semi-permissive (39°C) temperatures.

50-μL of competent cells were mixed with plasmid mixture and electroporated using the conditions described above, except 3-mL of SOC was added to the cells post-electroporation, then each transformation reaction was divided equally into three 1-mL aliquots that were incubated for 1 hour at 30°C or 39°C. Following the 1-hour recovery period, cells were serially diluted and plated as described above. Plates were incubated overnight at each respective temperature and the next day, viability and transformation efficiency was determined as described above.

DNA purification for 2D gel electrophoresis

Overnight cultures of cells containing pBR322 or pCL01 grown in LB supplemented with 15 μg/mL tetracycline or 20 μg/mL chloramphenicol, respectively, were diluted 100-fold into 10-mL of LB and grown to an OD_{600} of 0.5 at 37°C. A 0.75-mL aliquot of cells was then collected into an equal volume of ice-cold NET buffer (100 mM NaCl; 10 mM Tris, pH 8; 10 mM EDTA, pH 8), pelleted then flash frozen, and stored at -80°C.

For temperature-sensitive strains, overnight cultures containing each plasmid grown at the permissive temperature of 30°C were subcultured as described above and

grown to an OD_{600} of 0.3 in a shaking 30°C water bath. Half of the cells were then collected by filtration on 0.45-µm membranes (Fisher Scientific) and resuspended in media pre-warmed to either the semi-permissive (30°C) or non-permissive (43°C) temperatures, while the remaining culture was allowed to continue growing at 30°C. 0.75-mL aliquots of each culture were collected in an equal volume of ice-cold NET buffer immediately following resuspension in pre-warmed media and after a further 90 minutes of incubation at 30°C, 39°C or 43°C as described above.

Cell pellets were resuspended in 120-µL of lysis buffer (1 mg/mL lysozyme and 0.2 mg/mL RNaseA in 10 mM Tris, pH 8.0 1 mM EDTA, pH 8.0) then incubated at 37°C for 30 minutes. Then, 10-µL of 10 mg/mL Proteinase K and 10-µL of 20% Sarkosyl were added to each sample, and incubation was continued at 37°C for 1 hour. Samples were extracted once using four volumes of phenol-chloroform, followed by two volumes of chloroform, then dialyzed on a 47-mm Millipore 0.05-µm pore disk floating on 200-mL of 1 mM Tris, pH 8 1 mM EDTA, pH 8.0) for 30 minutes.

2D gel electrophoresis and Southern analysis

Purified DNA from cells containing the pBR322 plasmid were digested with the PvuII restriction enzyme, while purified DNA from cells containing the pCL01 plasmid were digested with the BstEII restriction enzyme. Following a 24-hour incubation at 37°C, digested DNA was extracted with one volume of chloroform then loaded onto a 0.4% agarose gel in 1x TBE and electrophoresed at 1 V/cm for 14-16 hours. For the second dimension, lanes were excised, rotated 90°, recast in a 1% agarose gel in 1x TBE, and electrophoresed at 6.5 V/cm for 6-8 hours.

DNA from the gels was transferred to Hybond N+ nylon membrane. Plasmid DNA was detected using either ^{32}P-labeled pBR322 or pCL01 plasmid DNA prepared by random-primer labeling (Agilent Technologies) using alpha-^{32}P-labeled-dCTP (3000 Ci/mmol), and visualized using a STORM PhosphorImager and analyzed using ImageJ software.

Sample preparation for replication profiles

Overnight cultures of each strain were diluted 100-fold into 10-mL of LB and grown to an OD_{600} of 0.5 at 37°C or 30°C for temperature-sensitive strains. Then, 0.75-mL aliquots of each culture were collected in an equal volume of ice-cold NET buffer (100 mM NaCl; 10 mM Tris, pH 8; 10 mM EDTA, pH 8). Cells were pelleted, flash frozen, and stored at -80°C.

For replication profiles following temperature shift in wild-type and temperature-sensitive strains, overnight cultures were diluted as described above and grown to an OD_{600} of 0.3 at the permissive temperature of 30°C. Half of the cells were then collected by filtration on 0.45-µm membranes (Fisher Scientific) and resuspended in media pre-warmed to either the semi-permissive (39°C) or non-permissive (43°C) temperature, while the remaining culture was allowed to continue growing at 30°C. 0.75-mL aliquots of each culture were collected in an equal volume of ice-cold NET buffer immediately following resuspension in pre-warmed media and after a further 90- and 180-minutes incubation at 30°C, 39°C or 43°C, as described above.

Stationary-phase wild-type cultures were grown in LB for 36 hours at 37°C for use as a normalization control.

Replication profiles

Genomic DNA was prepared as described for 2D gel electrophoresis above. Genomic DNA samples were sequenced using paired-end, 51-bp, bar-coded reads prepared and run using seqWell library prep kits (seqWell, Beverly MA, USA) and Illumina Next Seq 2000 (Illumina, San Diego, CA, USA). Breseq software is a command line tool that identifies mutations in DNA, relative to a reference genome {Deatherage and Barrick, 2014 #230134}. This software was used to identify mutations between each strain and the wild-type BW25113 reference genome. Sequencing reads were aligned to the reference genome using Bowtie 1.0.0 software. Aligned reads were characterized to determine the nucleotide frequency at each position. Sequences were divided into 1-kb bins and plotted using a custom Python script developed by our lab. Copy numbers for each strain were normalized against the wild-type stationary culture. Plots represent the relative copy numbers at each genomic location in 1-kb bins and depict the replication profile for each strain.

Table 2.1. Bacterial strains used in this study

Bacterial Strain	Genotype	Source
BW25113 Parent	(*araD-araB*)567, Δ*lacZ4787*(::*rrnB*-3), λ-, *rph*-1, Δ(*rhaD-rhaB*)568, *hsdR*514	(71)
CL4079	BW25113 *sbcD*::FRT-mini-kan *topA2103*	This study
CL5110	BW25113 *sbcD*::FRT *topA2103 ymjA*::FRT-mini-kan	This study
CL5111	BW25113 *topA2103 ymjA*::FRTminikan	This study
CL5250	BW25113 *recBCD*::kan	(24)
CL5328	N99 *gyrB*ts *yieL*::kan	This study
CL5379	BW25113 Δ(*topA-cysB*) *ymjA*::kan	This study
CL5380	BW25113 Δ(*topA-cysB*) *ymjA*::kan	This study
CL5427	N99 Δ(*topA-cysB*) *ymjA*::kan	This study
CL5428	BW25113 $parE^{ts}$::Tn10	
CL5429	BW25113 $parE^{ts}$ tet^{s}	This study
CL5431	BW25113 $gyrB^{ts}$ *yieL*::FRT-minikan	This study
JW1752	BW25113 *topB*::FRTminikan	(69)
JW1288	BW25113 *ymja*::FRTminikan	(69)
JW5612	BW25113 *yieL*::FRTminikan	(69)
RFM445	*rpsL galK2 gyrB221*(cou^{R}) *gyrB203*(Ts) Δ*trpE* Δ*lac74*	(31)
RFM475	*rpsL galK2 gyrB221*(cou^{R}) *gyrB203*(Ts) Δ*trpE* Δ(*topA-cysB*)*204* trp^{+} Δ*lac74*	(31)
parE(Ts)	MG1655 *parEts*::Tc	(72)

Table 2.2. Plasmids used in this study

Plasmids	Genotype	Source
pBR322	*amp*^{R} *tet*^{R} ColE1 origin	(74)
pCL01	*amp*^{R} *cam*^{R} λ origin	(20)

Chapter 3

Results

Topo I, but not Topo III, enhances the ability of replication forks to converge on plasmids

To better understand the role of DNA topoisomerases in completing DNA replication, we assessed the ability of mutants to maintain a plasmid that requires replication forks to converge. The 2-replisome plasmid, pCL01, utilizes a lambda origin of replication, loading dual helicases and the host's replication machinery (75, 76). This substrate has been previously used in our lab and we have shown that genes needed to maintain regions where replication forks converge are unable to do so when impaired (20, 24). As a control, the ability of the 2-replisome plasmid to replicate was compared relative to that of a 1-replisome plasmid, pBR322, which similarly uses the host machinery for transcription and replication, but only contains a single replisome and therefore replicates without a convergence event.

The transformation efficiency was determined by transforming a mixture containing both plasmids into wild-type and mutant strains (Fig 3.1A). If the topoisomerase is needed for the convergence of replication forks, we reasoned that the replication of the 2-replisome plasmid would be more severely impaired than that of the 1-replisome plasmid. As shown in Figure 3.1B, transformation of both the 1-replisome and 2-replisome plasmid was similar in the parental wild-type cells. By contrast, when the plasmid mixture was transformed into a *recBCD* mutant, the transformation frequency of the 2-replisome plasmid was severely reduced. As noted above, RecBCD is required to maintain the chromosomal region where replication forks converge, demonstrating that the 2-replisome

plasmid can be used to identify gene products participating in the completion reaction (7, 20).

To examine the type I DNA topoisomerases, we used deletion mutants of Topo I, *topA*, and Topo III, *topB*. However, most reports suggest Topo I is essential (40, 77), and we had difficulty constructing these mutants. We did obtain two separate isolates, which upon sequencing were found to have secondary, presumably suppressor mutations in addition to the *topA* deletion. The first isolate contained a cytosine to guanine transition mutation in *gyrB*, whereas the second isolate contained an approximately 900 kb duplication that encompassed *parE* and *parC.* Since the deletion was present and the strains remained viable, we continued characterizations of these strains. We also examined *topA2103* mutant, which was isolated as a spontaneous a one-base pair frameshift that removes the C-terminal domain but retains partial function. As seen in Figure 3.1B, transformation of the 2-replisome plasmid was severely impaired in *topA2103* and both *topA* deletion strains relative to the 1-replisome plasmid, suggesting that Topo I enhances the ability of replisomes to converge and complete. In contrast, no difference in the transformation frequency of the 2-replisome plasmid relative to the 1-replisome plasmid was seen in *topB* mutants, implying that its absence does not affect the reaction.

Topo IV, but not DNA gyrase, enhances the ability of replication forks to converge on plasmids

Both type II DNA topoisomerases are essential for viability; therefore, we used temperature sensitive *gyrB* and *parE* mutants to examine the type II topoisomerases. These mutants are viable at 30°C and are nonviable at 42°C (72, 78). We confirmed that both

mutants lost viability at the restrictive temperature when placed in our parental background. However, at 39°C, we found these mutants retained viability and used this temperature as a semi-permissive temperature for these assays. Following transformation of the plasmid mixture, all strains were grown at permissive or semi-permissive temperatures. As shown in Figure 3.2, at the permissive temperature, both 1-replisome and 2-replisome plasmids transformed into wild-type cells at similar frequencies, whereas transformants of the 1-replisome but not the 2-replisome plasmid were obtained in *recBCD* mutants, similar to that seen at 37°C. Additionally at the permissive temperature, the plasmids with or without convergent replisomes transformed *gyrB*^{ts} and *parE*^{ts} mutants at similar frequencies. However differences emerged at the semi-permissive temperature. Although *gyrB*^{ts} mutants remained capable of transformation by both 1- and 2-replisome plasmids; transformation of the 2-replisome plasmid in the *parE* mutant was severely impaired relative to the 1-replisome plasmid (Figure 3.2) demonstrating a requirement for Topo IV, but not DNA gyrase, to maintain plasmids containing convergent replisomes.

A temperature sensitive Topo IV mutant produces abnormal replication intermediates when two replication forks converge

The inability to transform plasmids containing 2-replisomes but not 1-replisome when Topo I or Topo IV function is compromised suggests that convergent replisomes create replication intermediates that cannot be processed in these mutants, leading to plasmid loss. Although transformants of 2-replisome plasmids in *topA* mutants could not be obtained, the ability to transform *parE*^{ts} mutants at permissive temperatures provides an opportunity to examine this question directly. 2-dimensional (2D) agarose gel

electrophoresis is a technique that can be used to visualize and differentiate the structure of replicating DNA fragments *in vivo* (79).

Non-replicating plasmids migrate as a linear fragment that forms the prominent spot observed on the 2D gels. Replicating fragments form structures that migrate more slowly because of their larger size and nonlinear shape. On plasmids with a unidirectional origin, the replicating fragments form Y-shaped molecules that migrate as an arc, extending out from the linear fragment (Figure 3.3A). Plasmids with a bidirectional origin, which contain two active replication forks, produce fragments with double-Y shapes. These molecules migrate as an inverted V-shape that extends up from the linear monomer fragment and down to the linear dimer fragment (Figure 3.3A).

In the case of the 1-replisome plasmid, both *gyrB*^{ts} and *parE*^{ts} mutants formed normal Y-shaped intermediates, similar to the parental strain. On plasmids containing 2-replisomes, *parE*^{ts} mutants leads to the formation of large, abnormal replication intermediates accumulating in these mutants. The formation of the abnormal intermediates were specific to cells with compromised Topo IV function, since they did not accumulate in the parental or *gyrB*^{ts} strains. The migration pattern of the abnormal intermediates in the *parE*^{ts} mutants are multimeric in size, having either one or two branch points. The multimeric size also implies that these molecules contain single-strand regions, which are resistant to digestion by the restriction endonuclease. This result demonstrates that suboptimal Topo IV activity impairs the ability to resolve and complete replication specifically on plasmids where replication forks converge.

Topo I and Topo IV are required to efficiently complete chromosome replication

The results above demonstrate that Topo I and Topo IV activity are required to maintain plasmid substrates that contain convergent replication forks. To determine if these enzymes function during the completion of replication on the chromosome, we used sequence frequency analysis to profile the genomes of replicating wild-type and mutant cultures. To profile replication on the chromosome, genomic DNA was extracted from growing cultures, fragmented, and prepared for high-throughput sequencing. The frequency of each sequence was observed then mapped to their position on the genome (Figure 3.4A). In actively growing wild-type cultures, the copy number of sequences surrounding the origin, which replicates first, will be higher than sequences further away from the origin, decreasing in frequency until reaching the terminus region, which replicates last. In *recBCD* mutants, a dramatic loss of sequences is observed in the region specifically where replication forks converge (Figure 3.4B), consistent with previous work suggesting this enzyme is required to join the strands of convergent forks {Courcelle et al., 2015 #89736; Hamilton et al., 2019 #299911}. When we examined the *topA* deletion strains, containing suppressors in DNA gyrase or Topo IV, we observed a modest increase in copy number, or over-replication, of the region where the replication forks converge (Figure 3.4C). This amplification was less prominent, but still detectable in the *topA2103* mutant. In contrast, the profile of the *topB* mutants appeared similar to wild-type cells. The results are consistent with those obtained with the 2-replisome plasmid and suggest that Topo I but not Topo III promotes the efficient completion of replication.

To examine the role of essential type II topoisomerases, temperature sensitive *gyrB* and *parE* mutants were grown at the 30°C then shifted to 39°C for 90 or 180 minutes before

the genomic DNA was purified and sequenced as described above. Under these conditions, replication in wild-type cultures has begun to slow down by the 90 minute time point and ceases replicating by 180 minutes, as the nutrients in the media are depleted and they enter stationary phase. In the replication profiles, this is observed as a decrease in difference between the frequency of origin and terminus sequences, as new initiations become less frequent by 90 minutes. By 180 minutes, new initiations have ceased and ongoing rounds of replication have completed, leaving equal frequencies at all regions around the chromosome. Following a shift to the semi-permissive temperature, the profile of the *gyrB*^{ts} mutants resembles and resolves in a manner similar to wild-type cultures with all sequence frequencies equally represented when replication in the cultures was complete. The *parE* replication profile, with 90 minutes of shifting to the semi-permissive timepoint, exhibits a modest over-replicated region at the loci where replication completes. By 180 minutes when replication has ceased, a dramatic degradation of the chromosome occurs, which begins at the site where completion occurs and progresses back to the origin. A similar profile can be seen in *recBCD* cultures that have ceased to replicate (data not shown). The results demonstrate a specific defect in completing replication when Topo IV activity is compromised. The extensive degradation that occurs argues that in the absence of Topo IV activity, the DNA ends of convergent forks are not joined and remain susceptible to exonucleolytic degradation, similar to that seen in *recBCD* mutants. Taken together, the plasmid and chromosomal observations imply that the activities of Topo I and Topo IV promote the efficient completion of replication when replication forks converge. When these activities are absent or impaired, cell death and genetic instabilities arise specifically at these loci.

Figures

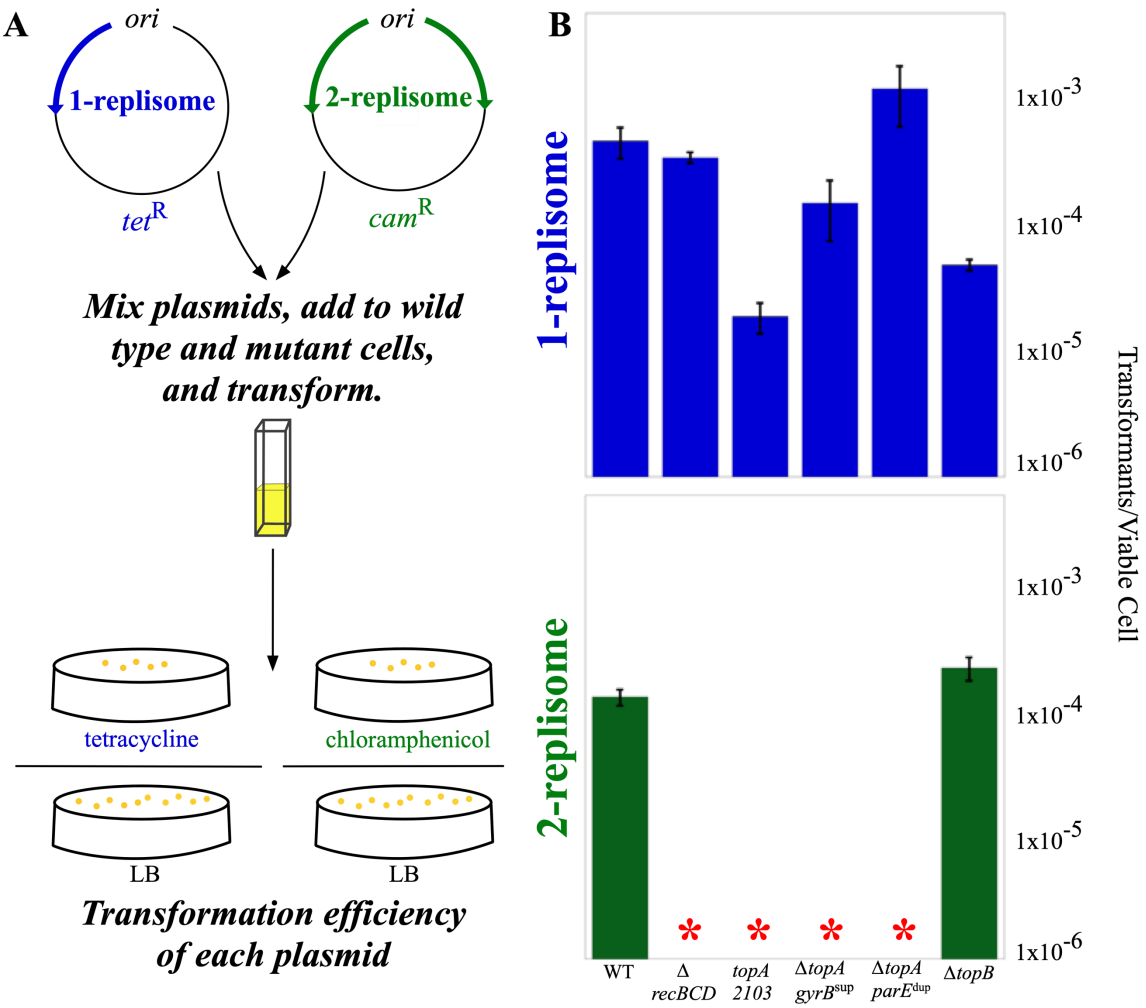

Figure 3.1. Type I DNA topoisomerases are required to maintain plasmids containing convergent replication forks.
A) A diagram showing how transformation frequencies were measured. Purified 1- and 2-replisome plasmid preparations were mixed and this mixture was transformed into wild-type and mutant strains, as indicated, before plating on selective and nonselective media.
B) Loss of Topo I activity impairs the ability to maintain plasmids containing convergent replication forks. The number of transformants per viable cell for the 1-replisome (pBR322) and 2-replisome (pCL01) is plotted for wild-type, Δ*recBCD, topA2103*, Δ*topA*, Δ*topA gyrB*^{sup}, Δ*topA parCE*^{dup}. *topA2103* contains a 1-base pair frameshift at base 2103 of the coding region. Δ*topA gyrB*^{sup} contains a C to G mutation at amino acid 630. Δ*topA parCE*^{dup} contains a duplication of a region encompassing *parC* and *parE.* Plots represent the average of at least three independent experiments. Error bars represent the standard error of the mean. Asterisks represent results that are below the limit of detection for this assay. Results indicated by asterisks suggest these mutants are defective in the replication of the plasmid.

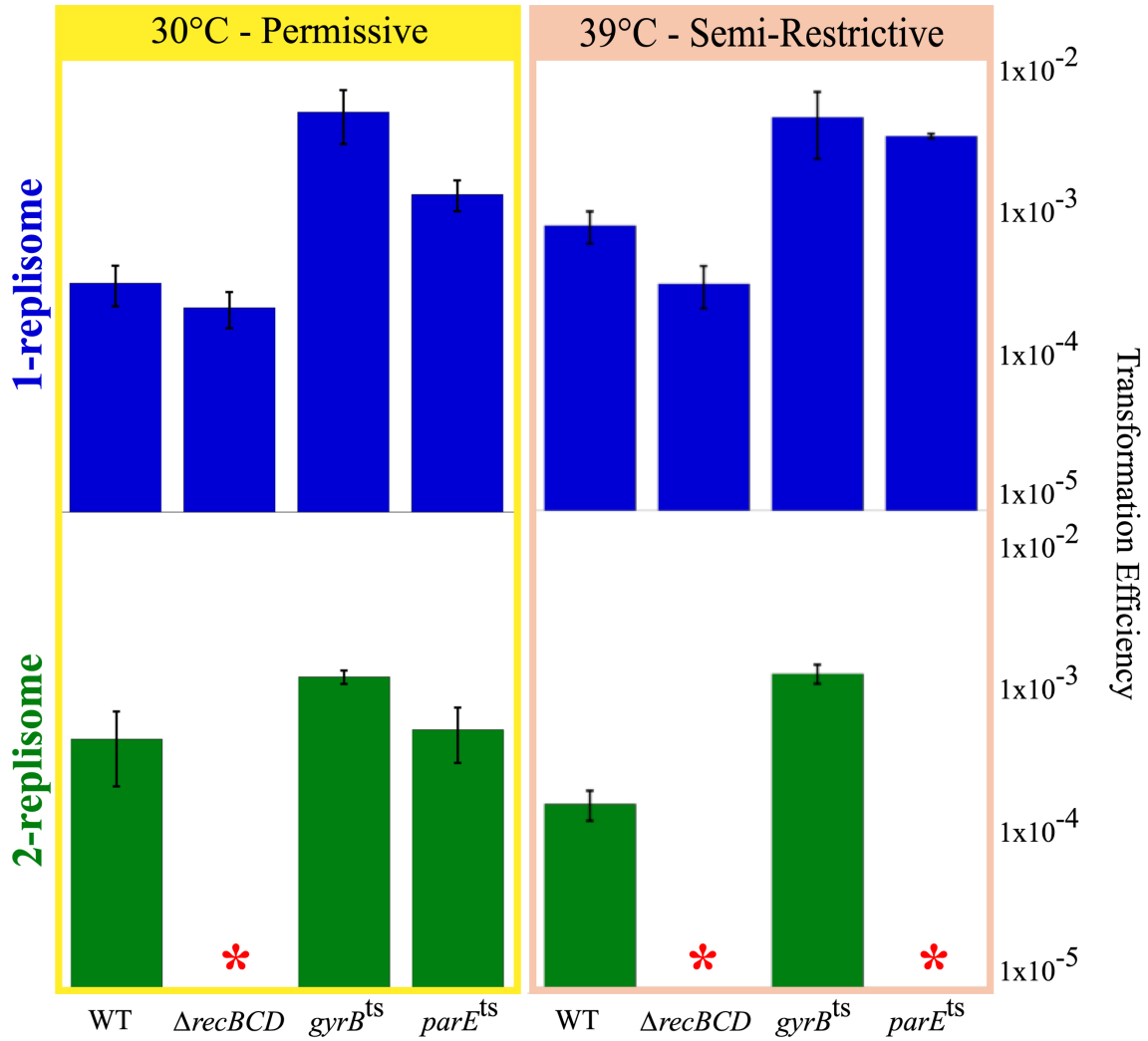

Figure 3.2. Topo IV is required to maintain plasmids with converging replication forks. Loss of Topo IV activity at the semi-restrictive temperature impairs the ability to maintain plasmids which contain convergent replication forks. The number of transformants per viable cell for the 1-replisome and 2-replisome plasmids is plotted at the permissive and semi-restrictive temperatures for wild-type, Δ*recBCD*, Δ*recBCD*, and temperature sensitive *gyrB* and *parE* mutants. Plots represent the average of at least three independent experiments. Error bars represent the standard error of the mean. Asterisks represent results that are below the limit of detection for this assay. Results indicated by asterisks suggest these mutants are defective in the replication of the plasmid.

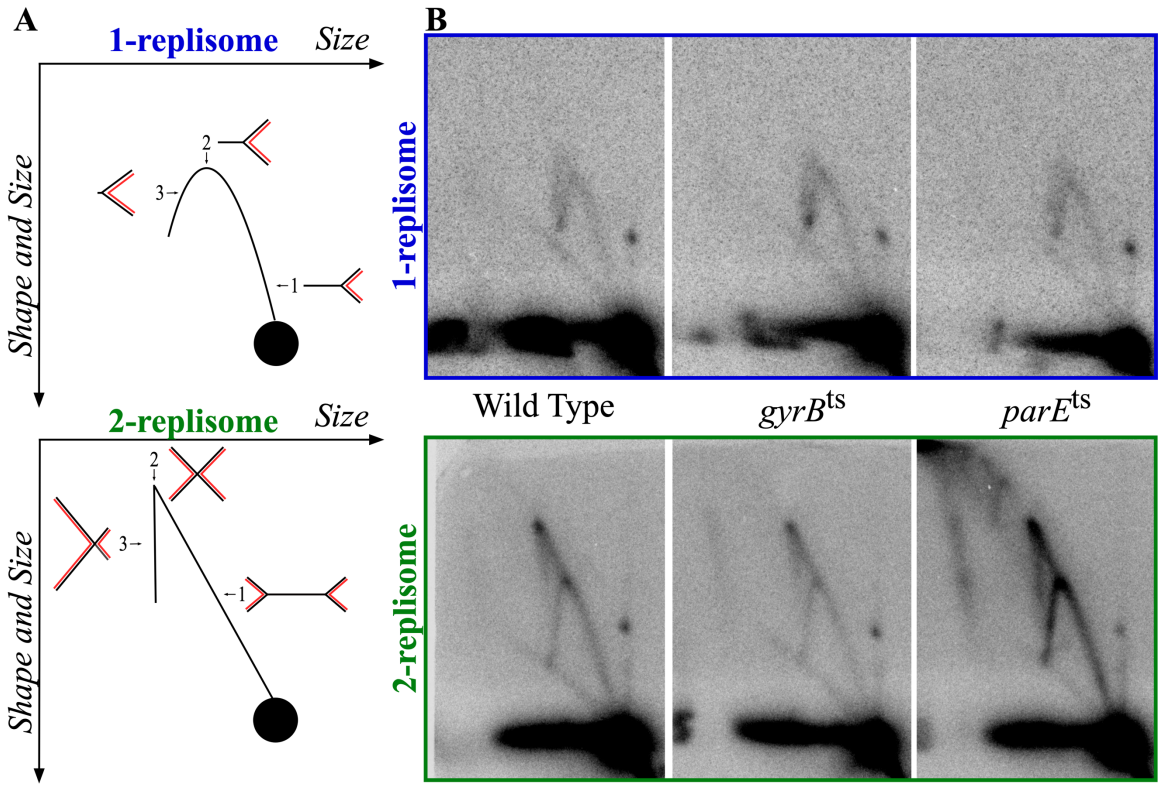

Figure 3.3. 2D gel electrophoresis of Topo IV mutants exhibit large, abnormal replication intermediates when replicating the 2-replisome plasmid.
A) Schematic of migration pattern for the 1- and 2-replisome plasmids. Replication intermediates are separated based on size in the first dimension and by shape in the second dimension. DNA molecules that are larger in size or more awkward in shape will not travel as far. Nonreplicating linear molecules remain at the bottom of the gel as a circular spot.
B) Loss of Topo IV activity leads to the formation of large, abnormal replication intermediates when replicating plasmids containing convergent replication forks. Plasmids were separately transformed into wild-type, *gyrB*^{ts} and *parE*^{ts} cells and 2D agarose gel analysis was subsequently completed on each strain. Abnormal replication intermediates are present in the *parE*^{ts} mutants. Representative gels of at least three independent experiments are shown.

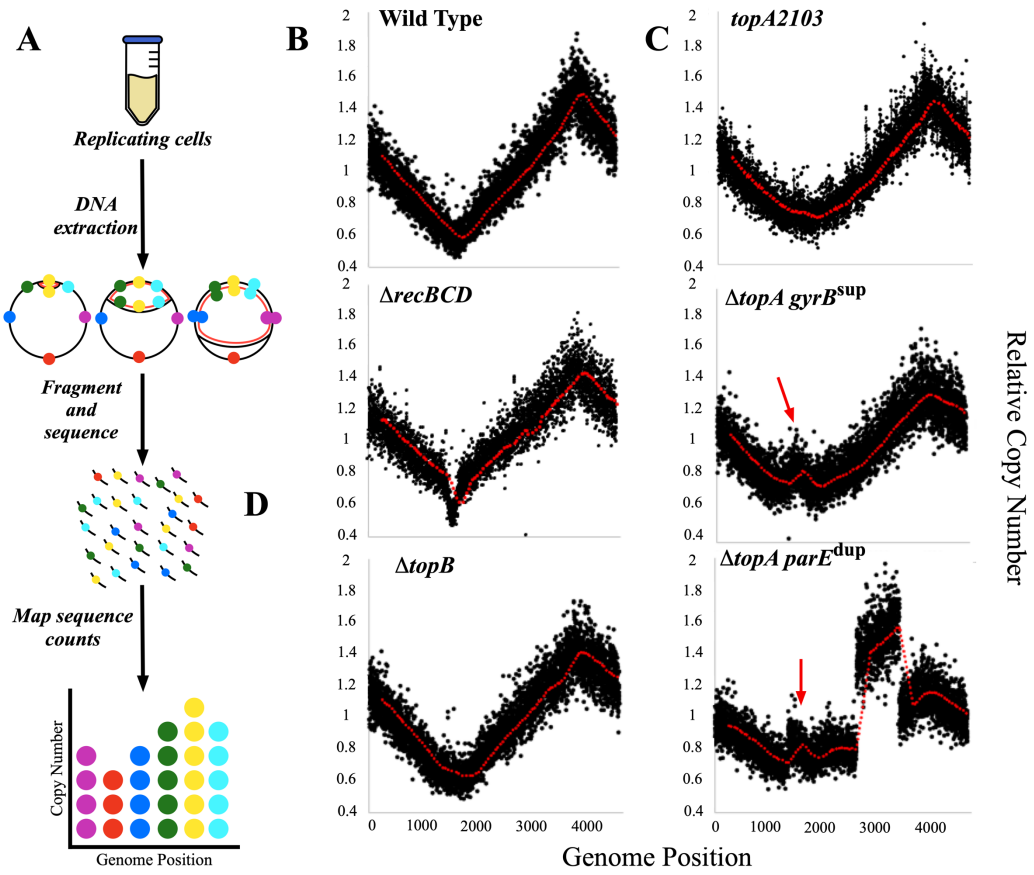

Figure 3.4. *topA* mutants exhibit overamplification in the terminus region of the chromosome.

A) A diagram showing how replication profiles were created. Genomic DNA was extracted and sequenced to map read numbers based on chromosome position. Wild-type replicating cultures will have the highest copy number at the origin and the lowest copy number at the terminus.

B) Controls used in replication profile assay. The parental strain and *recBCD* mutants were used as controls for this assay.

C) *topA* mutants exhibit an overamplification in the terminus region. *topA2103* does not lead to alterations within the terminus region of the chromosome. *topA* deletion mutants do exhibit an overamplification in the terminus region.

D) Topo III does not exhibit any chromosomal abnormalities.

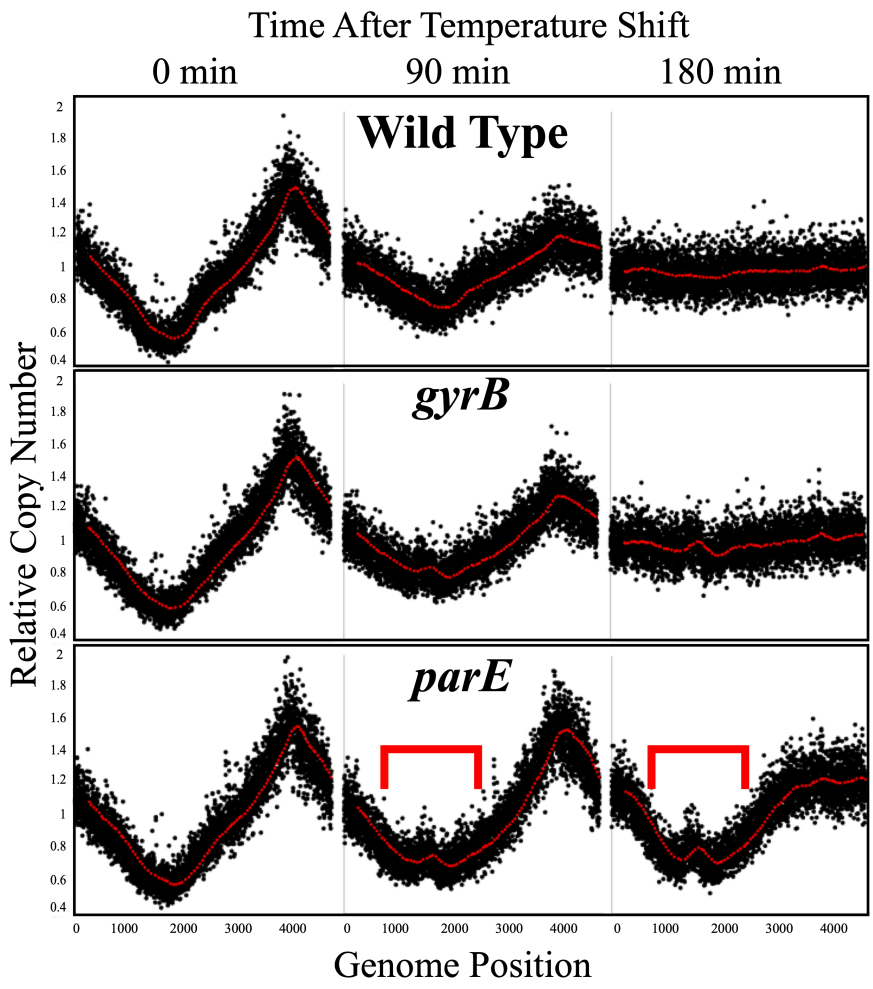

Figure 3.5. Topo IV mutants exhibit degradation in the terminus. Replication profiles were also created for type II topoisomerase mutants to determine if any abnormalities arise on the chromosomal level. As Topo IV continues to replicate after a temperature shift to the non-permissive temperature, Topo IV experiences genomic changes in the terminus region of the chromosome. Replication profiles of the parental and mutant strains were created as described above. By 180 minutes, there is significant degradation in the *parE* mutant. This degradation is not seen in the wild-type or DNA gyrase mutants.

Chapter 4

Discussion

The current model for the completion of replication is a multi-step pathway where replication forks converge and temporarily create an over-replicated region. This region of over-replicated DNA acts as a substrate SbcCD and RecBCD can process, which leads to an exact doubling of the sequences within the terminus region (5, 7, 8, 20). Here, I propose that Topo I and Topo IV play a role in the completion pathway. Based on biochemical characterizations of topoisomerases and data from this study, I propose that Topo I is needed to resolve topological constraints in order for the convergence of two replication forks to occur successfully. Once replication forks are able to successfully converge in the terminus region and after SbcCD-ExoI and RecBCD act, Topo IV acts to unlink the precatenated DNA and ensure the separation of the replicated chromosomes.

The role of Topo I in the completion of DNA replication

To better understand the role of Topo I in the completion of replication, I tested the ability of *topA* mutants to maintain plasmids with converging replication forks. *topA* mutants were defective in maintaining a 2-replisome plasmid, compared to the 1-replisome plasmid (Figure 3.1B). Once a plasmid effect was demonstrated in the *topA* mutants, I used marker frequency analysis to determine the effect of *topA* mutants on the chromosome. Marker frequency analysis demonstrated an overamplification in the terminus region of the chromosome (Figure 3.4C).

The over-replication seen in the terminus region of *topA* mutants suggests that the loss of Topo I activity leads to stable regions of DNA-RNA hybrids (R-loops) that lead to reinitiation events. This is based on the important role of Topo I in preventing the formation

of R-loops (80, 81). It has been well documented that the diminished activity of Topo I leads to an accumulation of negative supercoils leading to the formation of transcription-induced R-loops (31, 40, 40, 51, 82). Without the processing of these transcription-related negative supercoils, R-loops arise, creating genome instability (83). One way R-loops lead to genome instability is by acting as a source of illegitimate initiation. These sites of replication initiation from R-loops are known as *oriK* and the presence of an *oriK* in the terminus region has been previously demonstrated (48, 84, 85). The over-replication in the terminus region of the *topA* mutants supports this hypothesis.

The data presented in this study suggests that R-loop mediated reinitiation events can play a role in inhibiting the completion step of replication. This is supported by the deficiency in replicating the 2-replisome plasmid, compared to the 1-replisome plasmid. Additionally, the presence of an overamplification in the terminus region of *topA* mutants, suggests that R-loop mediated reinitiation events are specifically occurring in the terminus region. It can be inferred that this over-replicated region is acting as a block to the convergence of replication forks, potentially creating genomic instability within this region. Previous studies in mammalian cells have demonstrated that R-loop mediated genomic instability can affect replication (83, 86). Tuduri et al demonstrated an increase in fork stalling through the use of DNA combing in Top1-deficient mammalian cells (86). To demonstrate the connection between R-loops and fork stalling, researchers treated the cells with RNaseH, which degrades the RNA in R-loops, and found a reduction in fork stalling (86). The role of R-loops in causing genomic instability, through mechanisms such as inhibiting replication fork movement, has been well documented in bacteria as well as various eukaryotic model organisms (83, 87-89).

There are a couple of experimental methods that could be used to further support this R-loop hypothesis. Treating *topA* mutants with RNaseH could help determine if the over-replication seen in the terminus region is caused by R-loops. If R-loops are responsible for this over-replication, a possible effect of RNaseH treatment could be a lessening of the over-replicated DNA within the terminus region. Additionally, one could over-express *rnhA*, which encodes for RNase HI. It has been previously demonstrated that overexpression of RNase HI leads to the suppression of growth defects in *topA* mutants {Drolet et al., 1995 #179747}. This suggests that RNase HI may play a role in mediating the effects of R-loops in the absence of Topo I. If RNase HI is overexpressed, the formation of R-loops in the absence of Topo I may be decreased.

The results of this study suggest *topA* mutants are unable to complete DNA replication, which may provide better understanding as to why these mutants are inviable. There have been conflicting views on whether the accumulation of R-loops in *topA* mutants is what causes the severe growth defect seen in these mutants (77). Previous studies have demonstrated that overexpression of *rnhA*, which encodes RNase HI, can suppress the growth defects seen in *topA* mutants (31, 90). However, an *in vivo* study using a plasmid-based lethality assay to overexpress *rnhA* did not suppress *topA* growth defects, calling into question the direct effects of R-loops on the growth of *topA* mutants (77). The results in this study support the hypothesis that R-loop accumulation may be leading to genomic instability, preventing the completion of DNA replication and further growth of these mutants. This data is further supported by a previous study which tested the effect of RNase HI mutants on the completion step of replication (23). In this study, replication profiles of *rnhA* mutants exhibited an over-replication in the terminus region (23). This result further

supports my hypothesis that in the presence of R-loops, which would normally be resolved by RNase HI, over-replication in the terminus region prevents the convergence of replication forks from successfully occurring.

The role of Topo IV in the completion of replication

Results of this study also suggest that Topo IV is involved in the completion step of replication. Testing of the *parE*^{ts} mutants with the 2-replisome plasmid indicated that Topo IV helps with the completion reaction, potentially enhancing the convergence event between two replication forks. The replication profiles of the *parE*^{ts} mutants further supported our hypothesis that Topo IV is needed for the completion of replication and provided additional support that Topo IV primarily acts in the terminus region. This is demonstrated by the genomic degradation beginning in the terminus region and extending outward. There are no other regions of the replication profile that exhibit any abnormalities, which suggest that the terminus region is most affected in the absence of Topo IV.

The phenotypes of the Topo IV mutants tested in this study is the opposite of what was seen of the Topo I mutants, suggesting that these enzymes play important and unique roles in replication. Based on the data presented in this study and previous characterizations of Topo IV, I propose Topo IV acts to resolve precatenanes, which maintain the terminus region for the successful convergence of replication forks. The persistence of precatenanes in the absence of Topo IV is demonstrated in the 2D gel results of the Topo IV mutants. These results demonstrate the presence of large, abnormal replication intermediates, which can be inferred to be linked molecules that cannot be resolved without Topo IV. Additionally, the multimeric size indicates presence of single-strand regions. The degradation seen in the replication profile of these mutants suggests that the persistence of

precatenanes prevents nascent DNA ends from being joined, which leads to the persistence of single-stranded DNA regions. These single-stranded regions ultimately lead to genomic degradation.

The important ability of Topo IV to decatenate molecules by resolving precatenanes and catenanes has been repeatedly demonstrated in i*n vitro* studies (26, 29, 63, 91). The consequences of not resolving catenanes has been demonstrated in studies in yeast and other eukaryotic models, which have demonstrated similar phenotypes to the one seen in this study. A study using fluorescence microscopy to demonstrate the effects of TopIIα-deficiency in mouse cells saw irregular shaped nuclei in cells that had prematurely undergone cytokinesis (92). One proposal for the irregular shaped nuclei in these mice cells was the persistence of catenanes that led to irregular DNA replication. This same phenomenon of irregular replication leading to abnormal nuclei has been demonstrated in *top2* yeast mutants (93). Additionally, increased chromosome breakage in *top2* mutants in yeast has been demonstrated, which correlates with the genomic degradation seen in the Topo IV mutants of this study (94). Another study in yeast demonstrated an increase in double-strand breaks by separating genomic DNA from temperature sensitive Top2 mutants by pulse-field gel electrophoresis and analyzing replication intermediates by Southern blotting (95). This same study additionally used ChIP sequencing to determine Top2 binding sites and found that Top2 mainly interacts with termination regions of the yeast chromosome (95). These results suggest the same thing as the replication profiles of the Topo IV mutants in this study. The main chromosomal abnormality is seen in the terminus region of the chromosome, suggesting that Topo IV functionality is needed in the termination region of the bacterial chromosome.

Where Topo IV acts on the genome remains a topic of debate. There have been a mix of studies suggesting that Topo IV resolves precatenanes as replication occurs and allows for replisome progression. On the other hand, there have been studies that suggest Topo IV primarily works in the terminus region of the chromosome. An *in vitro* study compared the preferred substrates of DNA gyrase and Topo IV and suggested that Topo IV acts behind the replication fork to resolve precatenanes, while DNA gyrase acts in front of the fork (25). Additionally, a fluorescent microscopy study tagged Topo IV and a replisome-associated protein to monitor Topo IV positioning during replication (64). This study found Topo IV trailing behind the replisome-associated protein, suggesting it unlinks replicated DNA throughout replication (64). Another study measured cohesion time at different loci to demonstrate that precatenanes are the major source of cohesion in the cell (96). By increasing Topo IV concentration, they found a decrease in sister cohesion time and proposed that precatenanes were removed by Topo IV during replication (96).

On the other hand, there is compelling evidence which suggests Topo IV acts specifically within the terminus region of the chromosome. One study used ChiP sequencing on Flag tagged subunits of Topo IV to demonstrate that there is a strong Topo IV binding site at the *dif* site of the chromosome, suggesting Topo IV mainly acts within the terminus region of the chromosome (65). Another study demonstrated that the terminus region of the chromosome contains a major Topo IV cleavage site by stabilizing Topo IV-DNA complexes through ciprofloxacin treatment and sequencing the DNA after immunoprecipitation (66). This study identified a Topo IV cleavage site at the *dif* site of the chromosome that was approximately 10-fold greater in signal compared to other cleavage sites (66). Additionally, yeast two-hybrid analysis has helped characterized Topo

IV protein-protein interactions demonstrating that Topo IV interacts with FtsK, a protein that localizes within the terminus region and acts late in the cell cycle (97).

The data in this study supports previous evidence suggesting that the majority of unlinking by Topo IV occurs late in the cell cycle. If Topo IV was acting throughout replication, degradation throughout the chromosome would be expected, not just in the terminus region of the chromosome. Additionally, the presence of large, multimeric replication intermediates in the 2D gel of the Topo IV mutants supports the hypothesis that the majority of unlinking occurs towards the end of replication. This result suggests there is a build up of topological constraints that are not resolved by the absence of Topo IV.

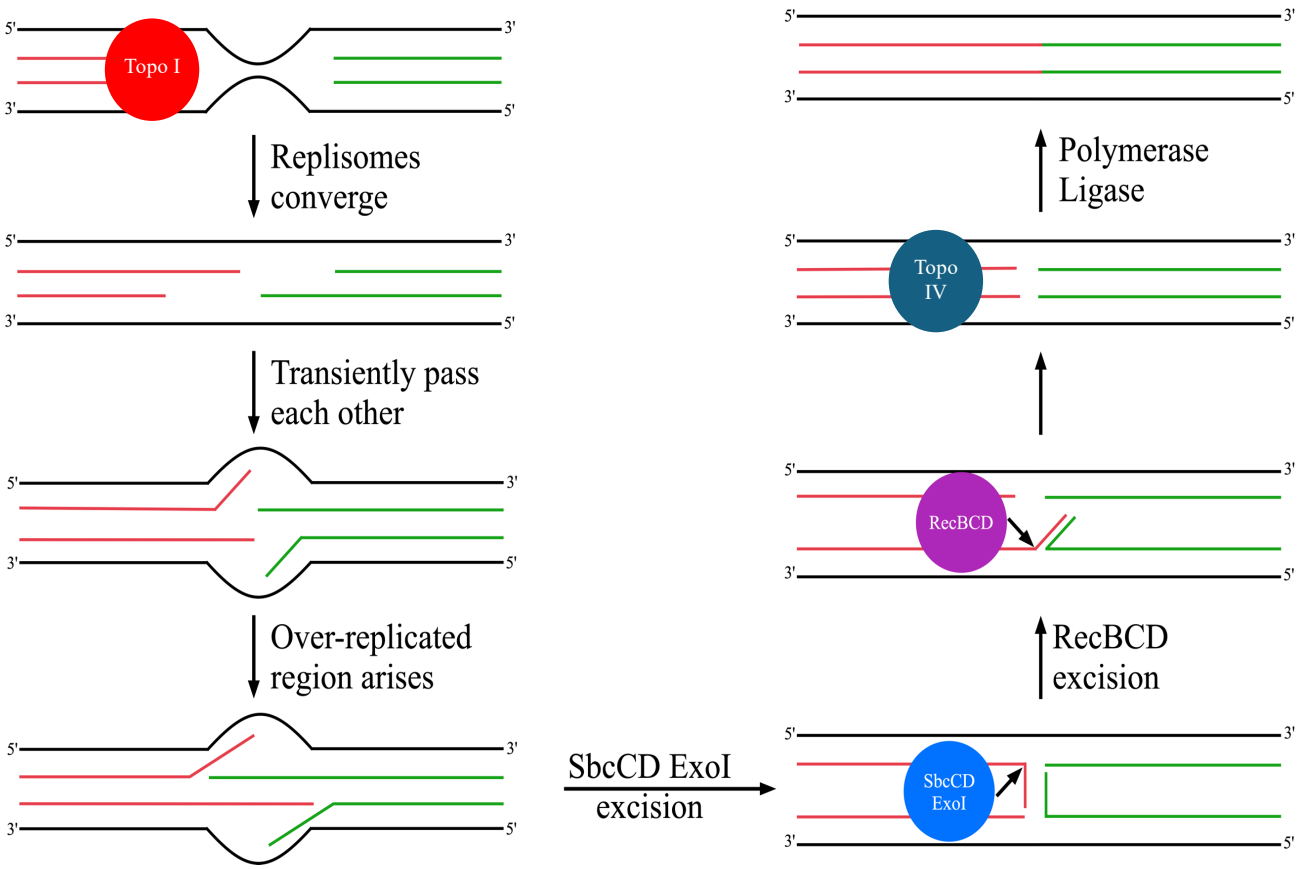

Figure 4.1. Proposed model of completion of replication with DNA topoisomerases. Topo I resolves negative supercoils, which allows for the convergence of replication forks to occur successfully. In the absence of Topo I, R-loops arise due to unresolved negative supercoils and can lead to overamplification in the terminus. The data presented in this study suggests this inhibits the convergence event of two replication forks. After the action of SbcCD-ExoI and RecBCD, Topo IV resolves precatenanes, which allows for the successful completion of replication. Without Topo IV, precatenanes are not resolved, which can lead to the persistence of free DNA ends and causing degradation in the terminus region.

Chapter 5

Appendix

One of the projects in the Courcelle lab, preceding my thesis project, focused on learning more about the SbcCD complex, a nuclease which is hypothesized to play a role in the completion step of DNA replication. This complex was first studied by testing a *sbcCD* double mutant using the plasmid assay. The 1-replisome and 2-replisome plasmids could replicate in the absence of both SbcC and SbcD. The next step was to determine if there was a separation of function between SbcC and SbcD so the single mutants were tested using the plasmid assay. In the absence of SbcD, the 1-replisome plasmid could replicate but the 2-replisome plasmid could no longer replicate. This result was unexpected, and a bit confusing, as the 2-replisome plasmid could replicate in the absence of both SbcC and SbcD. Because of this unexpected result, the *sbcD* single mutant was sent out for sequencing. Sequencing identified that this strain also contained a frameshift mutation in the *topA* gene. What was thought to be a *sbcD* single mutant was actually a *sbcD topA* double mutant.

My first task after joining the Courcelle lab was to determine which mutant was responsible for this unexpected phenotype. I separated the double mutant and created a *sbcD* single mutant and a *topA2103* single mutant. Both of these mutants were then tested using the plasmid assay. The *sbcD* single mutant could now replicate the 1-replisome and 2-replisome plasmids. This result aligned with our original expectations when studying the SbcCD complex. The *topA2103* single mutant could replicate the 1-replisome plasmid but could not replicate the 2-replisome plasmid. This indicated that this Topo I mutant

was responsible for the unexpected phenotype we saw when originally testing the single mutants of the SbcCD complex.

This new result suggested that Topo I plays a role in the convergence of replication forks on plasmids and led us to consider the role of topoisomerases in the completion step of replication and the topological structures that could possibly arise during this process. Learning about the different topological structures that arise during processes like replication and transcription (positive and negative supercoils), it seemed possible that topoisomerases could play a role in the completion step of DNA replication.

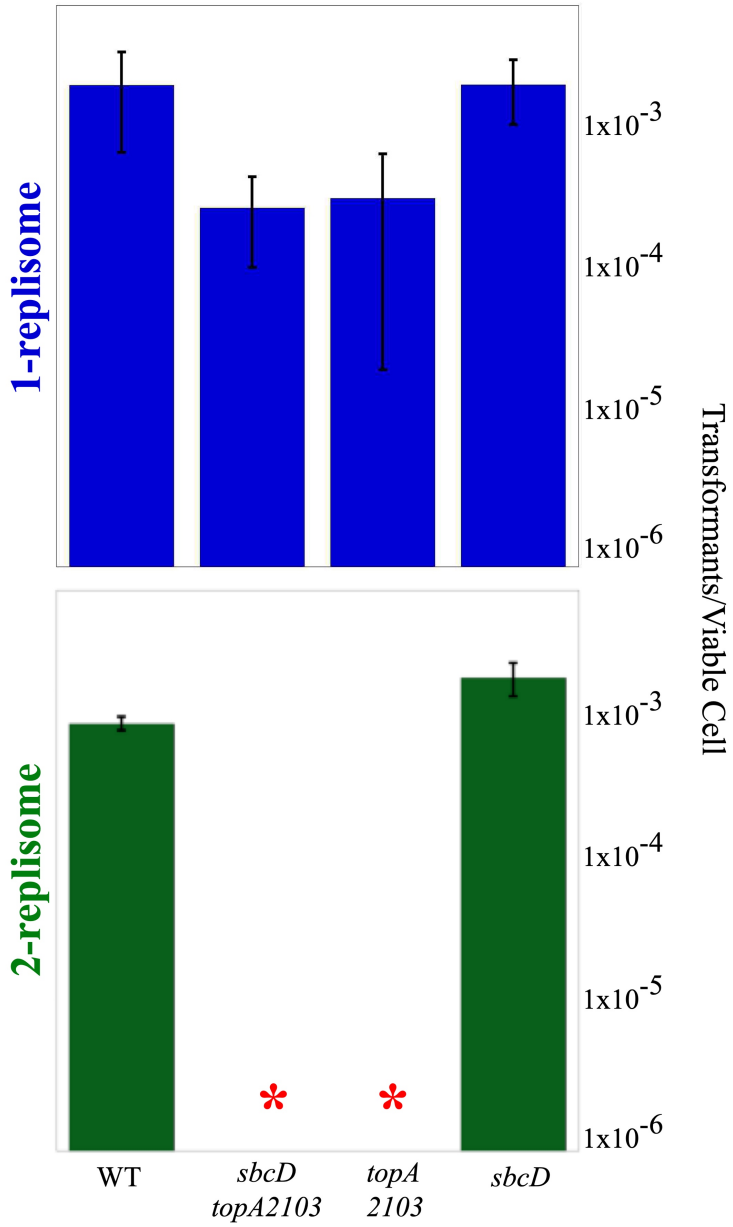

Figure 5.1. A *sbcDtopA2103* double mutant is unable to maintain converging replication forks, unlike the *sbcD* single mutant. The *sbcDtopA2103* strain was originally constructed as an *sbcD* single mutant. It was expected that this mutant would be capable of replicating plasmids containing converging replication forks. It was determined upon sequencing that this strain contains a frameshift mutation in the C-terminal domain of Topo I. Single mutants were constructed and used in the plasmid assay to determine that the *topA2103* mutant was responsible for this phenotype.

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