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# Genetic studies on the role of type IA DNA topoisomerases in DNA metabolism and genome maintenance in *Escherichia coli*

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Genetic studies on the role of type IA DNA topoisomerases in DNA metabolism and genome maintenance in *Escherichia coli*

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# Résumé

Le surenroulement de l'ADN est important pour tous les processus cellulaires qui requièrent la séparation des brins de l'ADN. Il est régulé par l'activité enzymatique des topoisomérases. La gyrase (*gyrA* et *gyrB*) utilise l'ATP pour introduire des supertours négatifs dans l'ADN, alors que la topoisomérase I (*topA*) et la topoisomérase IV (*parC* et *parE*) les éliminent. Les cellules déficientes pour la topoisomérase I sont viables si elles ont des mutations compensatoires dans un des gènes codant pour une sous-unité de la gyrase. Ces mutations réduisent le niveau de surenroulement négatif du chromosome et permettent la croissance bactérienne. Une de ces mutations engendre la production d'une gyrase thermosensible. L'activité de surenroulement de la gyrase en absence de la topoisomérase I cause l'accumulation d'ADN hyper-surenroulé négativement à cause de la formation de R-loops. La surproduction de la RNase HI (*rnhA*), une enzyme qui dégrade l'ARN des R-loops, permet de prévenir l'accumulation d'un excès de surenroulement négatif. En absence de RNase HI, des R-loops sont aussi formés et peuvent être utilisés pour déclencher la réplication de l'ADN indépendamment du système normal *oriC*/DnaA, un phénomène connu sous le nom de « constitutive stable DNA replication » (cSDR).

Pour mieux comprendre le lien entre la formation de R-loops et l'excès de surenroulement négatif, nous avons construit un mutant conditionnel *topA rnhA gyrB*(Ts) avec l'expression inductible de la RNase HI à partir d'un plasmide. Nous avons trouvé que l'ADN des cellules de ce mutant était excessivement relâché au lieu d'être hypersurenroulé négativement en conditions de pénurie de RNase HI. La relaxation de l'ADN a été montrée comme étant indépendante de l'activité de la topoisomérase IV. Les cellules du triple mutant *topA rnhA gyrB*(Ts) forment de très longs filaments remplis d'ADN, montrant ainsi un défaut de ségrégation des chromosomes. La surproduction de la topoisomérase III (*topB*), une enzyme qui peut effectuer la décaténation de l'ADN, a corrigé les problèmes de ségrégation sans toutefois restaurer le niveau de surenroulement de l'ADN. Nous avons constaté que des extraits protéiques du mutant *topA rnhA gyrB*(Ts) pouvaient inhiber l'activité de surenroulement négatif de la gyrase dans des extraits d'une souche sauvage,

suggérant ainsi que la pénurie de RNase HI avait déclenché une réponse cellulaire d'inhibition de cette activité de la gyrase. De plus, des expériences *in vivo* et *in vitro* ont montré qu'en absence de RNase HI, l'activité ATP-dépendante de surenroulement négatif de la gyrase était inhibée, alors que l'activité ATP-indépendante de cette enzyme demeurait intacte. Des suppresseurs extragéniques du défaut de croissance du triple mutant *topA rnhA gyrB*(Ts) qui corrigent également les problèmes de surenroulement et de ségrégation des chromosomes ont pour la plupart été cartographiés dans des gènes impliqués dans la réplication de l'ADN, le métabolisme des R-loops, ou la formation de fimbriae.

La deuxième partie de ce projet avait pour but de comprendre les rôles des topoisomérases de type IA (topoisomérase I et topoisomérase III) dans la ségrégation et la stabilité du génome de *Escherichia coli*. Pour étudier ces rôles, nous avons utilisé des approches de génétique combinées avec la cytométrie en flux, l'analyse de type Western blot et la microscopie. Nous avons constaté que le phénotype Par$^-$ et les défauts de ségrégation des chromosomes d'un mutant *gyrB*(Ts) avaient été corrigés en inactivant *topA*, mais uniquement en présence du gène *topB*. En outre, nous avons démontré que la surproduction de la topoisomérase III pouvait corriger le phénotype Par$^-$ du mutant *gyrB*(Ts) sans toutefois corriger les défauts de croissance de ce dernier. La surproduction de topoisomérase IV, enzyme responsable de la décaténation des chromosomes chez *E. coli*, ne pouvait pas remplacer la topoisomérase III. Nos résultats suggèrent que les topoisomérases de type IA jouent un rôle important dans la ségrégation des chromosomes lorsque la gyrase est inefficace.

Pour étudier le rôle des topoisomérases de type IA dans la stabilité du génome, la troisième partie du projet, nous avons utilisé des approches génétiques combinées avec des tests de « spot » et la microscopie. Nous avons constaté que les cellules déficientes en topoisomérase I avaient des défauts de ségrégation de chromosomes et de croissance liés à un excès de surenroulement négatif, et que ces défauts pouvaient être corrigés en inactivant *recQ*, *recA* ou par la surproduction de la topoisomérase III. Le suppresseur extragénique *oriC15::aph* isolé dans la première partie du projet pouvait également corriger ces problèmes. Les cellules déficientes en topoisomérases de type IA formaient des très longs

filaments remplis d'ADN d'apparence diffuse et réparti inégalement dans la cellule. Ces phénotypes pouvaient être partiellement corrigés par la surproduction de la RNase HI ou en inactivant *recA*, ou encore par des suppresseurs isolés dans la première partie du projet et impliques dans le cSDR (*dnaT18::aph* et *rne59::aph)*. Donc, dans *E. coli*, les topoisomérases de type IA jouent un rôle dans la stabilité du génome en inhibant la réplication inappropriée à partir de *oriC* et de R-loops, et en empêchant les défauts de ségrégation liés à la recombinaison RecA-dépendante, par leur action avec RecQ.

Les travaux rapportés ici révèlent que la réplication inappropriée et dérégulée est une source majeure de l'instabilité génomique. Empêcher la réplication inappropriée permet la ségrégation des chromosomes et le maintien d'un génome stable. La RNase HI et les topoisomérases de type IA jouent un rôle majeur dans la prévention de la réplication inappropriée. La RNase HI réalise cette tâche en modulant l'activité de surenroulement ATP-dependante de la gyrase, et en empêchant la réplication à partir des R-loops. Les topoisomérases de type IA assurent le maintien de la stabilité du génome en empêchant la réplication inappropriée à partir de *oriC* et des R-loops et en agissant avec RecQ pour résoudre des intermédiaires de recombinaison RecA-dépendants afin de permettre la ségrégation des chromosomes.

**Mots-clés** : Surenroulement, RNase HI, R-loops, gyrase, ATP, topoisomérases de type IA, topoisomérase I, topoisomérase III, ségrégation des chromosomes, RecA, RecQ Abstract

# Abstract

DNA supercoiling is important for all cellular processes that require strand separation and is regulated by the opposing enzymatic effects of DNA topoisomerases. Gyrase uses ATP to introduce negative supercoils while topoisomerase I (*topA*) and topoisomerase IV relax negative supercoils. Cells lacking topoisomerase I are only viable if they have compensatory mutations in gyrase genes that reduce the negative supercoiling level of the chromosome to allow bacterial growth. One such mutation leads to the production of a thermosensitive gyrase (*gyrB*(Ts)). Gyrase driven supercoiling during transcription in the absence of topoisomerase I causes the accumulation of hypernegatively supercoiled plasmid DNAs due to the formation of R-loops. Overproducing RNase HI (*rnhA*), an enzyme that degrades the RNA moiety of R-loops, prevents the accumulation of hypernegative supercoils. In the absence of RNase HI alone, R-loops are equally formed and can be used to prime DNA replication independently of *oriC*/DnaA, a phenomenon known as constitutive stable DNA replication (cSDR).

To better understand the link between R-loop formation and hypernegative supercoiling, we constructed a conditional *topA rnhA gyrB*(Ts) mutant with RNase HI being conditionally expressed from a plasmid borne gene. We found that the DNA of *topA rnhA gyrB*(Ts) cells was extensively relaxed instead of being hypernegatively supercoiled following the depletion of RNase HI. Relaxation was found to be unrelated to the activity of topoisomerase IV. Cells of *topA rnhA gyrB*(Ts) formed long filaments full of DNA, consistent with segregation defect. Overproducing topoisomerase III (*topB*), an enzyme that can perform decatenation, corrected the segregation problems without restoring supercoiling. We found that extracts of *topA rnhA gyrB*(Ts) cells inhibited gyrase supercoiling activity of wild type cells extracts *in vitro*, suggesting that the depletion of RNase HI triggered a cell response that inhibited the supercoiling activity of gyrase. Gyrase supercoiling assays *in vivo* as well as in crude cell extracts revealed that the ATP dependent supercoiling reaction of gyrase was inhibited while the ATP independent relaxation reaction was unaffected. Genetic suppressors of a triple *topA rnhA gyrB*(Ts) strain that

restored supercoiling and corrected the chromosome segregation defects mostly mapped to genes that affected DNA replication, R-loop metabolism and fimbriae formation.

The second part of this project aimed at understanding the roles of type IA DNA topoisomerases (topoisomerase I and topoisomerase III) in chromosome segregation and genome maintenance in *E. coli*. To investigate the role of type IA DNA topoisomerases in chromosome segregation we employed genetic approaches combined with flow cytometry, Western blot analysis and microscopy (for the examination of cell morphology). We found that the Par- phenotypes (formation of large unsegregated nucleoid in midcell) and chromosome segregation defects of a *gyrB*(Ts) mutant at the nonpermissive temperature were corrected by deleting *topA* only in the presence of *topB*. Moreover, overproducing topoisomerase III was shown to correct the Par- phenotype without correcting the growth defect, but overproducing topoisomerase IV, the major cellular decatenase, failed to correct the defects. Our results suggest that type IA topoisomerases play a role in chromosome segregation when gyrase is inefficient.

To investigate the role of type IA DNA topoisomerases in genome maintenance, in the third part of the project, we employed genetic approaches combined with suppressor screens, spot assays and microscopy. We found that cells lacking topoisomerase I suffered from supercoiling-dependent growth defects and chromosome segregation defects that could be corrected by deleting *recQ*, *recA* or overproducing topoisomerase III and by an *oriC15::aph* suppressor mutation isolated in the first part of the project. Cells lacking both type 1A topoisomerases formed very long filaments packed with diffuse and unsegregated DNA. Such phenotypes could be partially corrected by overproducing RNase HI or deleting *recA*, or by suppressor mutations isolated in the first part of the project, that affected cSDR (*dnaT18::aph and rne59::aph*). Thus, in *E. coli*, type IA DNA topoisomerases play a role in genome maintenance by inhibiting inappropriate replication from *oriC* and R-loops and by preventing RecA-dependent chromosome segregation defect through their action with RecQ.

The work reported here reveals that inappropriate and unregulated replication is a major source of genome instability. Preventing such replication will ensures proper chromosome segregation leading to a stable genome. RNase HI and type IA DNA topoisomerases play a leading role in preventing unregulated replication. RNase HI achieves this role by modulating ATP dependent gyrase activity and by preventing replication from R-loops (cSDR). Type IA DNA topoisomerases ensure the maintenance of a stable genome by preventing inappropriate replication from *oriC* and R-loops and by acting with RecQ to prevent RecA dependent-chromosome segregation defects.

**Keywords** : Supercoiling, RNase HI, R-loops, gyrase, ATP, type I A topoisomerases, topoisomerase I, topoisomerase III, chromosome segregation, genome maintenance, RecA, RecQ.

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# Abbreviations

ATP: Adenosine triphosphate

ADP: Adenosine diphosphate

aph: Aminoglycoside phosphotransferase gene cassette

bp: base pairs

cSDR: constitutive Stable DNA Replication

DAPI: 4', 6-diamidino-2-phenylindole

$^{o}$C: Degrees Celsius

Δ: Deletion

DNA: Deoxyribonucleic acid

DUE: DNA unwinding element

*E. coli*: *Escherichia coli*

EOP: efficiency of plating

Fig: Figure

h: Hours

iSDR: Inducible Stable DNA Replication

IPTG: Isopropyl β-D-1-thiogalactopyranoside

kan$^{r}$: Kanamycin resistance

kb: kilo base

LB: Luria-Bertani

Lk: Linking number

µg: Microgram

µl: Microliters

µM: Micromolar

ml: Milliliters

mM: Millimolar

OD: Optical density

*oriC:* Origin of replication C

*oriK*: Origin of replication K

Par$^{-}$: Partitioning defective phenotype

PCR: Polymerase chain reaction

RPA: Replication protein A

RNase: Ribonuclease

Rif: Rifampicin

SSB: Single- Stranded DNA Binding protein

NaCl: Sodium chloride

SDR: Stable DNA Replication

σ: Supercoiling density

Ts: Temperature sensitive

UV: ultraviolet

# Dedication

This thesis is dedicated to the memory of my late father, Paa Usongo Isaac Akuta. His guidance and encouragement was crucial in my life. He initiated this process and I wish he was here to witness it. I miss him every day and I take solace in that he is resting in Gods kingdom. Thanks to mom, Ogwe Ruth Usongo for being there for me.

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# CHAPTER 1: Literature review

## 1.1. Historical perspective of the DNA entanglement problem

The double helical structure of DNA described by Watson and Crick took the scientific community by storm and projected them to instant fame. In their model termed the double helix model, the two strands are interwound and follow a right hand helical path around a central axis and run in opposite directions with a helical repeat of 10 bp per turn. The two strands are held together by base pairing through the formation of hydrogen bonds with thymine pairing with adenine and cytosine pairing with guanine (Watson & Crick, 1953b). In regards to the base pairing in the double helix, Watson and Crick stated that "It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material" (Watson & Crick, 1953b). The concept of complementarity that they predicted in their model suggested that a parent DNA molecule could be duplicated by copying each strand in tandem with the Watson and Crick base pairing (Watson & Crick, 1953a).

The interwound nature of the helix imposes a strict requirement on the mechanism of replication. The parental strands need to be unlinked for the separation of daughter chromosomes to occur. Watson and Crick acknowledged the challenge but stated that it was "not insuperable" (Watson & Crick, 1953b). This admission by Watson and Crick was not enough to dissuade criticism of their model. Among them Max Delbruck raised the concern on how the semi-conservative scheme of DNA replication (an appellation designated since it was envisioned that one half of the progeny duplex was inherited and the other half newly synthesized) could be successfully executed without tangling of the progeny DNA, with the parental DNA spelling disastrous consequences for the cell following division. He went ahead to propose solutions involving breakage every 1/2 turn of the helix during replication and suggested an alternate model of discontinuous DNA replication to avoid the linking problem (Delbruck, 1954). The confirmation that DNA replicated semi-conservatively (Meselson & Stahl, 1958) implied that the entanglement problem was real.

In addition, the famous autoradiographic images (Cairns, 1963b) of *E. coli* showing a circular chromosome that replicates semi-conservatively from a unique region of the parent DNA ring (Cairns, 1963a) equally proved the entanglement problem.

Further solidifying the entanglement problem was the discovery that the DNA of the polyomavirus that infects animals was a double stranded ring with intact strands (Weil & Vinograd, 1963). This observation further complicated the issue of separating the DNA strands which became a topological problem. Another observation that added a piece to the puzzle was the discovery that polyoma DNA existed in varying forms namely "extended cyclic form" and "tightly coiled cyclic form" or supercoiled DNA (Vinograd et al., 1965). The observation that DNA from polyomavirus was negatively supercoiled triggered more structural studies (Vinograd et al., 1965) which explained the varying forms of DNA observed through a quantity called the linking number (Lk). Lk is the number of times one strand crosses the other and it is the sum of two terms twist (Tw) which is the local winding of the two strands around each other, and writhe (Wr) a measure of DNA supercoiling (White, 1969). Tw and Wr are related to Lk by the simple equation: Lk = Tw +Wr (White, 1969).

It was soon deduced that a DNA ring from a natural source would display a value of LK lower than that of the same ring in its most stable structure under typical experimental conditions used in the laboratory. This quantity termed the linking number of a relaxed DNA ring has a symbol $LK_0$. A DNA ring with a linking number Lk is said to be negatively supercoiled if $Lk < LK_0$, or $(Lk- LK_0) < 0$ or positively supercoiled if $Lk > LK_0$ or $(Lk-LK_0) > 0$ (J. C. Wang, 1974). In this regard, the polyoma DNA rings from natural sources were thus defined to be negatively supercoiled and the supercoiled state of the DNA was viewed as a compensation for the torsional stress produced by a reduction in Lk (Vinograd et al., 1965).

Proper duplication and transmission of genetic information from one generation to another warrant that the linking number must be reduced to zero. It is in this light that a prominent topologist named William Pohl rejected the plectonemic Watson-Crick model for a paranemic (side-by-side) structure of DNA, for he could not imagine how a global property like the Lk could be nulled enzymatically (Pohl & Roberts, 1978). The quest to know why DNA from natural sources was negatively supercoiled lead to the discovery of

DNA topoisomerases as nature's solution for DNA strand unlinking and modulation of the torsional state of the duplex (Wang, 1971, 1985).

### 1.1.1. DNA supercoiling

As mentioned earlier, supercoiling in a DNA molecule can either be positive or negative. Literally speaking, DNA is said to be negatively supercoiled when it has a deficiency in the linking number compared with the relaxed DNA. Because negatively supercoiled DNA has fewer helical turns than the molecule would normally contain as a linear or relaxed molecule, it is said to be underwound (Sinden, 1994). The consequence of this underwinding in the number of helical turns is that there are more base pairs per helical turn, which leads to a decrease in the angle of twist between the adjacent base pairs. Underwinding thus creates torsional tension following the winding of the double helix. This torsional tension drives the supertwisting of the molecule forming a right-handed (or clockwise) supercoil (Sinden, 1994). On the contrary, positively supercoiled DNA is overwound in terms of the number of helical turns, leading to fewer bases per helical turn and thus an increase in the winding angle between the adjacent base pairs, which equally create torsional tension in the winding of the helix. The tension in the winding helix is relieved by the positive supercoiling of the DNA forming a left-handed (counterclockwise) supercoil (Sinden, 1994).

DNA isolated from natural sources is negatively supercoiled (Vinograd et al., 1965). In fact DNA supercoiling has been shown to be a target of evolutionary selection for mutations that lead to an overall increase in negative supercoiling have been identified in evolutionary experiments. Mutations in genes such as *topA* encoding topoisomerase I (topo I) and *dusB* which regulates the expression of *fis* (encoding the histon-like protein Fis ) have been identified in long term evolutionary experiments (Crozat et al., 2010). Several lines of experimental evidence have confirmed that negative supercoiling causes the double helix to adopt a branched and plectonemic or interwound structure. Non-viscous nucleoids obtained by treating cells with lysozyme (enzymes that hydrolyses bacterial cell walls) and mild ionic detergent (for cell lysis) and analyzed by sedimentation and electron

microscopy reveals an interwound DNA structure (Giorno, Hecht, & Pettijohn, 1975; Kavenoff & Bowen, 1976; Worcel & Burgi, 1972). Plasmid DNA structure produced by recombination with the lambda integrase and analyzed with sensitive and non-disruptive topological tests have confirmed the presence of interwound DNA *in vivo* (Bliska & Cozzarelli, 1987).

Supercoils do not only exist as interwound supercoils. Negative supercoils can also physically exist as left-handed toroidal coils whereby the DNA is wrapped around proteins.

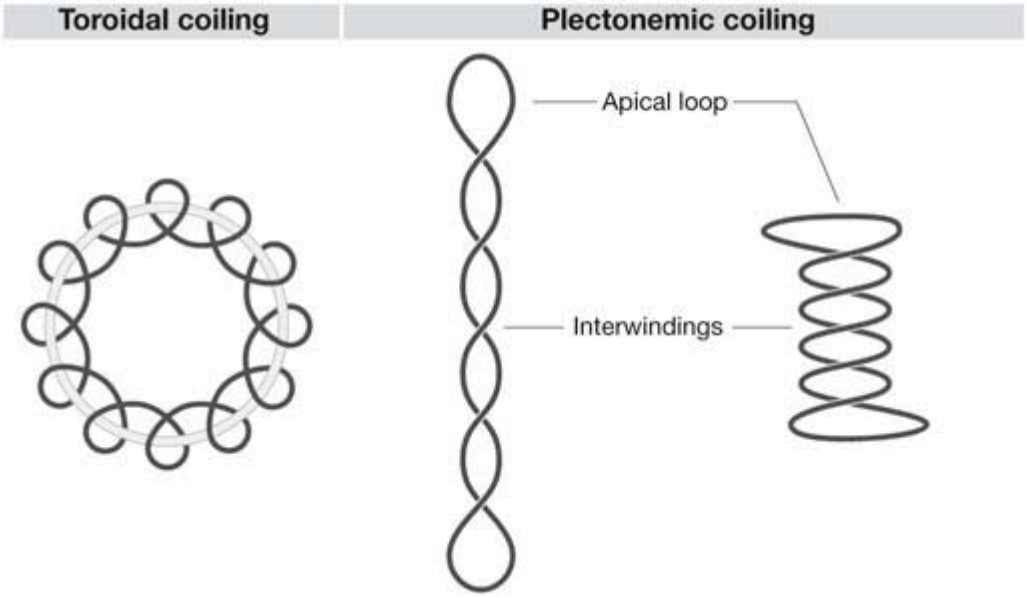


**Figure 1. Illustration of the geometry of toroidal and plectonemic supercoils**. DNA depicting a single form of toroidal DNA and two geometries of plectonemes which have the same superhelical density. Conversion of the long plectoneme to the shorter one is achieved by DNA bending and untwisting of the DNA duplex (Travers & Muskhelishvili, 2007). Figure used with permission from Nature publishing group.

In solution, supercoils are manifested as a mixture of interwound and toroidal coils. Studies have shown that for DNA in bacteria about 70 % of the linking number deficiency is distributed as writhe change and about 30 % is distributed as a change in twist (Boles, White, & Cozzarelli, 1990). Toroidal supercoils are very important for the biological organization of the nucleosome core in eukaryotes that involves the toroidal coiling of the DNA around proteins. In this organization, the supercoiling energy is restrained by this

wrapping. In bacteria, studies have shown that the dominant form of supercoiled DNA is a plectoneme (Crisona et al., 1999). In fact, measurements using the cross-linking agent trimethyl-psoralen which forms interstrand abducts on DNA have revealed that most of the DNA in bacteria exists as unconstrained plectonemic supercoils *in vivo*, whereas little if any of the DNA in eukaryotic nucleus is in this form (Sinden, Carlson, & Pettijohn, 1980). More importantly it has been found that in the nucleosome, DNA is marginally overtwisted (White, Cozzarelli, & Bauer, 1988; Zivanovic et al., 1988) whereas in the plectonemic form when free in solution, negatively supercoiled DNA is undertwisted significantly (Boles et al., 1990).

Undertwisting facilitates processes that require strand separation and this explains the importance of negative supercoiling in cellular processes such as initiation of DNA replication (Alexandrov et al., 1999; Pruss & Drlica, 1989), positive or negative modulation of transcription of numerous bacterial genes (Lim et al., 2003; Peter et al., 2004; Travers & Muskhelishvili, 2005b), chromosome segregation (Holmes & Cozzarelli, 2000; Sawitzke & Austin, 2000; Usongo et al., 2008), and recombination (Alexandrov et al., 1999; Crisona et al., 1994; Hatfield & Benham, 2002). Even though cellular processes, especially replication and transcription, generate strong torsional forces along the DNA axis leading to the buildup of transient supercoiling in both the positive and negative directions (Liu & Wang, 1987; Lockshon & Morris, 1983), homeostatic regulation by DNA topoisomerases (to be discussed ahead) resets the topological status into an underwound state (Travers & Muskhelishvili, 2007; Zechiedrich et al., 2000).

### 1.1.2. Global supercoiling

The importance of DNA supercoiling in *E. coli* has no yard stick measurement as it is implicated in numerous cellular transactions as stated below. Because of its importance, a modest reduction of negative supercoiling in bacteria is lethal (Zechiedrich, Khodursky, & Cozzarelli, 1997). In addition, supercoiling is equally fragile as just a single break in DNA can relax the entire chromosome killing the cell since free negative supercoiling is necessary for viability (Gellert et al., 1976). The process of DNA replication also causes

relaxation because of the presence of gaps in the lagging strand. Fortunately for the cell, these doom day scenarios are avoided thanks to the intuitive organization of the chromosome. Actively replicating parts of the chromosome and any insults be it physical, chemical or enzymatical to the DNA backbone are confined to isolated regions of the chromosomes that are topologically independent and protect the bulk of the chromosome from the dangers of relaxation. These regions are called domains (Postow et al., 2004). Topological domains are defined as regions of the DNA that are topologically constrained at their ends and are thus independent with regards to the entire chromosome. Global supercoiling is defined as the average superhelical density of all supercoiling domains. The superhelical density ($\sigma$) is controlled in a very narrow range and deviations from this range in either direction are growth inhibitory (DiNardo et al., 1982). Mathematically, $\sigma$ can be defined as $\Delta LK/LK_0$ (Vologodskii & Cozzarelli, 1994). $LK_0 = N/\gamma$ where N is the number of base pairs in the molecule and $\gamma$ is the mean number of base pairs per turn in the double helix under a given set of conditions, $\Delta LK = LK - LK_0$ (Vologodskii & Cozzarelli, 1994). DNA extracted from cells has a $\sigma$ between -0.03 and -0.09 (Bauer, 1978). In *E. coli*, genetic studies using DNA topoisomerase mutants have established that for vigorous growth to occur, global supercoiling must lie within a $\pm$15% range of supercoiling (Drlica, 1992).

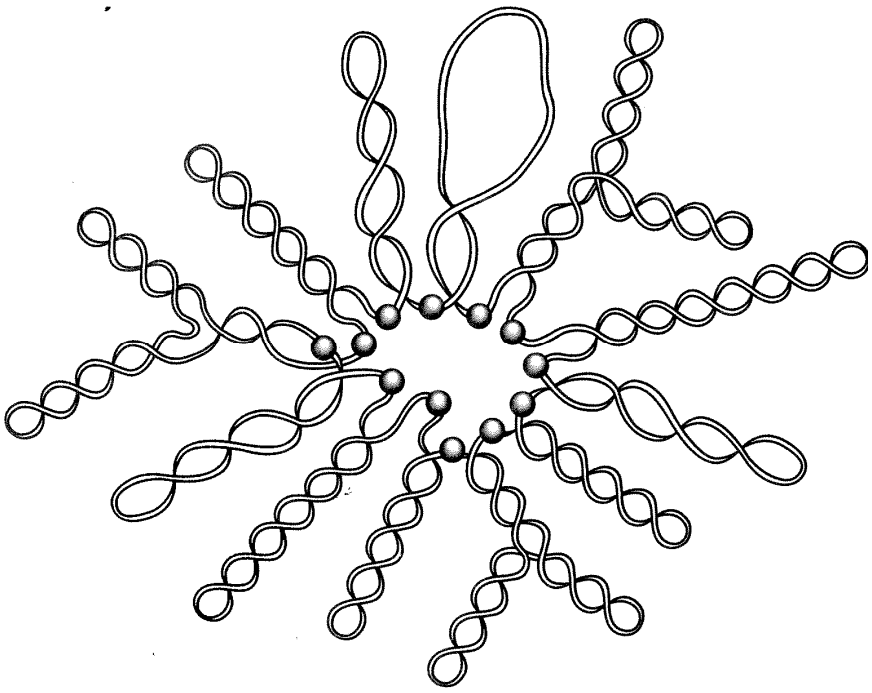

**Figure 2. Schematic representation of topological domain organization of the *E. coli* chromosome.** The *E. coli* chromosome is organized into independent topological domains *in vivo*. The shaded spheres represent the domain boundaries (Sinden, 1994). Figure used with permission from Elsevier.

Evidence of the existence of domains has been documented. Chromosomes isolated from *E. coli* are found to be folded by unconstrained negative supercoiling and several nicks are required to relax the entire chromosome (Delius & Worcel, 1974; Worcel & Burgi, 1972). The conclusion from these papers is that the *E. coli* chromosome is divided into no more than 50 topological domains with an average length of about 100 kb. By counting the number of supercoiled loops visible by electron microscopy, domains were estimated to be between 65 and 200 per nucleoid (Kavenoff & Bowen, 1976; Kavenoff & Ryder, 1976). Another method that is used to estimate the number of domains per nucleoid is psoralen photobinding to bacterial chromosomes partially relaxed by DNA breaks induced with X-rays *in vivo*. This method estimates the number of domains to be around 50 (Sinden & Pettijohn, 1981). More recent experiments employing less invasive approaches have shown that domain sizes are much smaller. A study in *S. enterica* examining the

topological requirements of site specific recombinases concluded that topological domains average 25 kb in length (Higgins et al., 1996).

A recent less invasive method to determine the size of domains has been developed (Postow et al., 2004) and it is based on the fact that negative supercoiling can modulate the activity of specific promoters by either increasing or decreasing their output (McGovern et al., 1994; Travers & Muskhelishvili, 2005b; Willenbrock & Ussery, 2004). This method takes advantage of the 306 supercoiling sensitive genes that are distributed widely on the genome and respond rapidly and reliably to supercoil relaxation. After DNA relaxation, 106 of the supercoiling sensitive genes (SSGs) become induced for transcription while 200 genes are repressed (Peter et al., 2004). In this method, double strand breaks were introduced onto specific locations on the chromosome by controlling the *in vivo* expression of restriction enzymes. The measure of the distance from a SwaI site to the promoter of a supercoiling sensitive gene (SSG) was combined with microarray expression patterns before and after cleavage with restriction enzymes. Following the analysis of the SSG transcription data, a model was proposed which predicts that the *E. coli* chromosome consists of variable loops and random distribution of domain barriers with an average domain size of 10 kb, significantly smaller than the previously estimated domain sizes (Postow et al., 2004).

Small domains confer several advantages to the cell. Small domains make life easy for decatenating enzymes by concentrating catenane links, making global processes more local (Espeli et al., 2003). By concentrating catenane links, the free energy of catenation will also increase and this will help drive the decatenation reaction to completion (Vologodskii & Cozzarelli, 1993). Catenanes and precatenanes will easily be resolved following replication in small domains (Schvartzman & Stasiak, 2004). Domain independence implies that each domain is shielded from the other by a barrier. Despite the importance of domain barriers little is known about the cellular components of these barriers *in vivo*. For bacteria, *in vitro* studies with proteins such as FtsK and SpoIIIE found that they are able to constrain DNA loops (Aussel et al., 2002; Bath et al., 2000; Pease et al., 2005). Moreover, these proteins are anchored in the bacterial inner membrane, making

them likely to form topological barriers on chromosomes that they act upon. Stable RNAs or transcriptional complexes can also stabilize chromosomes. RNases can decondense isolated chromosomes and these decondensed chromosomes contain a high fraction of nascent mRNA and DNA-bound RNA polymerase (Pettijohn et al., 1970; Stonington & Pettijohn, 1971; Worcel & Burgi, 1972).

The role of transcription in domain barrier formation has also been tested with the transcriptional inhibitor rifampicin. Although rifampicin causes chromosomes to decondense upon isolation (Dworsky & Schaechter, 1973; Pettijohn & Hecht, 1974), there is no change in domain numbers *in vivo* (Sinden & Pettijohn, 1981). In bacteria, since transcription and translation are coupled, insertion of the nascent polypeptide into the membrane in the case of mRNA encoding membrane bound proteins can also act as a domain barrier and this has been shown using plasmid DNA (Lynch & Wang, 1993). The role of transcription-translation coupling in domain barrier formation has also been demonstrated in the *Salmonella typhimurium* chromosome (Deng, Stein, & Higgins, 2004). A more recent *in vivo* study identified H-NS, Fis and transketolase (TktA) as domain barrier proteins and these proteins play a role in the supercoiling of domains by forming topological barriers in the chromosomes (Hardy & Cozzarelli, 2005).

### 1.1.3. Local supercoiling

Cellular processes such as DNA replication and transcription that involve tracking of huge protein complexes along double stranded DNA can transiently cause local perturbations in DNA topology. Evidence implicating transcriptional activity with DNA supercoiling has been documented. Highly positively supercoiled pBR322 DNA has been extracted from *E. coli* treated with gyrase inhibitors (Lockshon & Morris, 1983). pBR322 DNA extracted from *topA* mutants of *E. coli* and *S. typhimurium* harbors a high degree of negative supercoiling and this depends on the transcription of the *tetA* gene (Pruss & Drlica, 1986).

These observations led Liu and Wang (Liu & Wang, 1987) to propose the twin supercoiled domain model of transcription. The basis of this model is that elongating RNA polymerase molecules cannot rotate freely around the double-helical DNA because of the bulk of the polymerase with associated nascent transcripts as well as attached ribosomes. As a result, the DNA is forced to rotate upon itself. If the end of the DNA molecules are constrained in some way either by being very long or attached to cellular structures, polymerase tracking will cause the DNA ahead of the transcription complex to be overwound and the DNA behind to be underwound. Thus, polymerase movement will generate domains of positive supercoiling ahead of its passage and domain of negative supercoiling behind it. This model has been supported experimentally both *in vitro* and *in vivo* (Drolet, Bi, & Liu, 1994; Leng, Amado, & McMacken, 2004; Leng & McMacken, 2002; Rovinskiy et al., 2012).

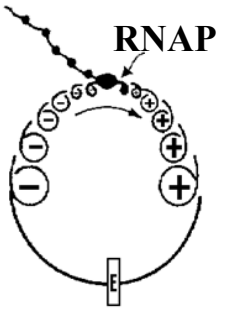


**Figure 3. Twin supercoiled domain model:** Transcription generates domains of positive and negative supercoils respectively in front and behind of the transcription complex represented by RNAP. E. depicts the frictional barrier against the rotation of the duplex around it helical axis (Wu et al., 1988). Figure adapted with authorization from Elsevier.

Even though it has been shown that topoisomerases are able to relax transcription induced supercoiling (Cook et al., 1992; Drolet, 2006; Massé & Drolet, 1999b; Rovinskiy et al., 2012), some studies have shown that irrespective of the presence of topoisomerases, localized supercoiling exists and can be exploited to trigger a variety of important DNA transactions (Bowater, Chen, & Lilley, 1994; Dunaway & Ostrander, 1993; Figueroa & Bossi, 1988; Kouzine & Levens, 2007; Kouzine et al., 2004; Kouzine et al., 2008; Ljungman & Hanawalt, 1992). It has also been found that depending on transcription

intensity and the disposition of topoisomerases, local supercoiling may exceed the relaxation activity of topoisomerases, forcing the residual DNA torsional stress to propagate through the surrounding DNA (referred to as dynamic supercoiling) (Kouzine et al., 2008).

The double helix which is in the predominant B-form (the most common form of DNA inside the cell and the one described by Watson and Crick) could adopt, depending on the sequence composition, a variety of alternative structures. In order to form these structures, the DNA duplex must be melted and this can be achieved by a high level of negative supercoiling (Bloomfield, Crothers, & Tinoco, 1974). In humans cells, dynamic supercoiling has been measured *in vivo* and *in vitro* by identifying chromosomal regions that have sequences susceptible to the formation of non-B DNA structures, and these regions are found to be mostly located upstream of active promoters (Kouzine et al., 2004; Kouzine et al., 2008). Dynamic supercoiling has been shown to persist for some time irrespective of the presence of normal concentrations of functional topoisomerases in the cell (Kouzine et al., 2008) and this suggests that topoisomerases are unable to immediately keep up or prevent the build-up of transient torsional stress induced by transcription. The non-B DNAs produced as a result of dynamic supercoiling have been shown to be bound by a variety of proteins that can change DNA conformation, implying that these non-B DNAs structure are important to the cell (Brooks, Kendrick, & Hurley, 2010; Kouzine & Levens, 2007).

The importance of non-B-DNA in gene regulation is illustrated by the human *c-myc* proto-oncogene. The protein c-Myc is a crucial regulator of up to 15 % of human genes, and this protein is essential for cell homeostasis, differentiation and growth (Liu et al., 2006). If not properly regulated, *c-myc* becomes a lethal oncogene that plays a role in many cancers (Hanahan & Weinberg, 2011). Because both the c-Myc mRNA and c-Myc protein are too short-lived to provide an effective feedback mechanism, the cell has evolved an alternative feedback mechanism that uses DNA dynamics to ensure regulation of *c-myc* transcription. This is provided by a 90 bp far upstream element (FUSE) of the human c-*myc* gene. The transcriptional activity of the *c-myc* promoter is enhanced by the supercoiling sensitive FUSE sequence. This sequence is sensitive to negative supercoiling. Elevated

levels of negative supercoiling melts this sequence. This in turn enables this sequence to bind to the transcription activator FUSE- binding protein (FBP). FBP increases promoter activity by interacting with the general transcription factor TFIIH to drive *c-myc* transcription. Another protein called the FBP interacting repressor (FIR) binds to FBP and FUSE and represses *c-myc* transcription (Liu et al., 2006). Therefore FUSE melting acts as a sensor for transcription to provide either positive feedback (via FBP) or negative feedback (via FIR) in the regulation of *c-myc* transcription.

Another important conformational sequence involved in *c-myc* regulation is the CT-element located 250 bp upstream of the main promoter (Brooks & Hurley, 2009; Siddiqui-Jain et al., 2002). This element has been shown to adopt non-B structures in supercoiled DNA, *in vitro* as well as *in vivo* (Kohwi & Kohwi-Shigematsu, 1991; Michelotti et al., 1996). The transcriptional activator Sp1 binds the CT element in its normal B-DNA structure to activate transcription. It has also been suggested that this sequence can also adopt a single-stranded conformation due to supercoiling accumulation generated by transcription. It is in this single-stranded form that the transcription factors hnRNPK and CNBP bind to maintain the active state of transcription (Brooks & Hurley, 2009; Michelotti et al., 1995; Tomonaga & Levens, 1996). The CT element can also adopt a non-B DNA structure conformation and in this conformation, the binding sites of the transcription factors are sequestered, leading to transcriptional silencing (Sun & Hurley, 2009).

Transcription induced local supercoiling also plays a role in the regulation of gene expression. A well-documented example is the activation of the *S. typhimurium leu-500* promoter. It has been found that a point mutation in this promoter that confers leucine auxotrophy is phenotypically suppressed by a mutation in the gene coding for topoisomerase I (Lilley & Higgins, 1991). This point mutation is located in the -10 region, making open complex formation energetically expensive, and the absence of topo I would energetically favor open complex formation at the mutant promoter. However, placing this mutant promoter on an extra chromosomal plasmid even in the absence of topo I (Richardson, Higgins, & Lilley, 1988) does not reproduce the same effect, thereby implicating local rather than global change in template topology. In addition, the *leu-500* promoter can be activated in a topo I negative background when it is placed divergent to the

tetracycline gene (*tetA*) on a plasmid (Chen et al., 1992). Activation only occurs upon transcription and translation of the *tetA g*ene. Thus, the level of supercoiling that is generated is sufficient to induce open complex formation on the *leu-500* promoter located upstream.

### 1.1.4. Constrained versus unconstrained supercoiling

A topological domain can harbor two sorts of supercoils: constrained and unconstrained supercoils (Cozzarelli & Wang, 1990). Though hidden, constrained supercoils are not lost when DNA is nicked (Pettijohn & Pfenninger, 1980). The portion of the overall supercoiling that is lost when DNA is nicked is called unconstrained supercoiling or superhelical tension. In *E. coli,* about 50% of the DNA supercoils are free (Bliska & Cozzarelli, 1987). Thus, about 50 % of supercoils are constrained by proteins binding to DNA (Bensaid et al., 1996; Bliska & Cozzarelli, 1987; Pettijohn & Pfenninger, 1980; Sinden & Kochel, 1987). Although a major source of this restraining comes from proteins that separate DNA strands as well as proteins of the replication apparatus and RNA polymerases, architectural proteins such as heat-stable-nucleoid-structuring protein (H-NS), integration host factor (IHF), the histone-like HU proteins, and the factor for inversion stimulation (FIS) have also been implicated (Drlica & Rouviere-Yaniv, 1987; Travers & Muskhelishvili, 2005a). Even though the exact role and mode of DNA interactions of these proteins are not entirely clear, some clues are beginning to emerge. The HU heterodimer not only constrains negatively supercoiled DNA by stabilizing plectoneme folds, it may also wrap DNA into a left-handed nucleosome-like structure (Guo & Adhya, 2007). HU deficient mutant strains have been shown to exhibit levels of supercoiling 15 % lower than those of wild-type strains (Hsieh, Rouviere-Yaniv, & Drlica, 1991). The stabilization of negative supercoils by HNS is likely achieved by interacting with DNA crossings. H-NS and IHF mutants show a high reduction in negative supercoiling compared to the wild-type (Higgins et al., 1988; Parekh, Sheridan, & Hatfield, 1996). The factor for inversion stimulation (FIS) binds at crossovers in plectonemes (Schneider et al., 2001) but it can also locally stabilize a DNA toroid (Maurer et al., 2006). In eukaryotes, the majority of

supercoils are restrained by nucleosomal organization following the toriodal wrapping of DNA around histones (Holmes & Cozzarelli, 2000).

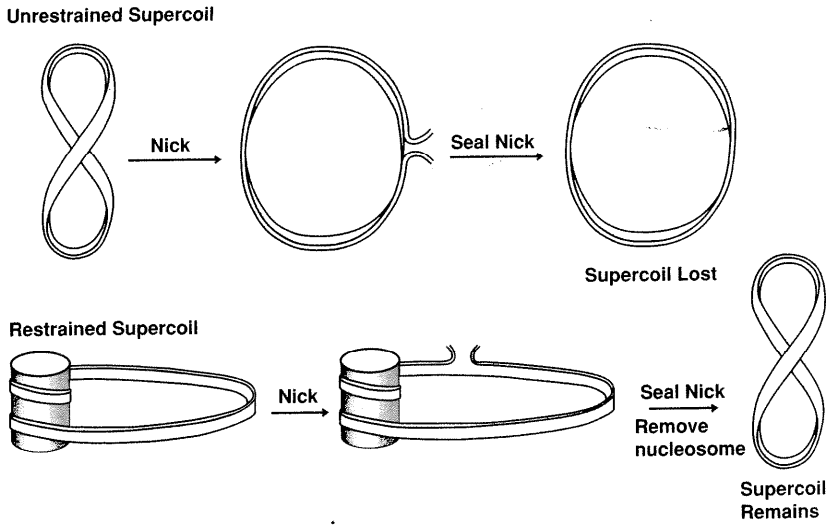


**Figure 4.Constrained and unconstrained supercoils.** In the unrestrained supercoil upper figure, any insult on the DNA backbone leading to chromosome breakage will lead to complete DNA relaxation. In the constrained supercoil represented below, supercoils are constrained by proteins and, in the event of strand breakage, supercoils are not lost since they are constrained. Figure obtained from (Sinden, 1994). Figure used with permission from Elsevier.

## 1.2. DNA topoisomerases

The choice was made by nature early in evolution to have the plectonemic DNA double helix as the carrier of genetic information. From this initial choice, the challenge to duplicate DNA became apparent and this was deeply rooted in the plectonemic nature of the DNA helix. However, this challenge was no match to nature's remedy, DNA topoisomerases. They evolved alongside DNA to solve the topological problems associated with it. Their universal partnership with DNA was blessed by nature from the beginning. Equipped with the ability to cut, shuffle and religate DNA strands, topoisomerases can add or remove supercoils, untangle interlocked double stranded DNA segments (catenanes)

and introduce or remove knots from DNA rings (Hartman et al., 2013; Wang, 2002). The dexterity of these enzymes at solving topological puzzles is achieved via the simple and elegant chemistry of transesterification (Wang, 2002). It is through this mechanism that the strand breakage reaction of topoisomerases is achieved. The first step is the attack of the DNA phosphorus by tyrosyl oxygen of the enzyme, thus forming a covalent phosphotyrosine link and at the same time breaking a DNA phosphodiester bond. Through a second transesterification reaction, which is the reverse of the first, the DNA strands are rejoined. In the reverse reaction, basically the oxygen from the DNA hydroxyl group that is generated from the first reaction attacks the phosphorous of the phosphotyrosine link and as a result, the covalent bond between the protein and DNA is broken and the DNA backbone bond is reformed (Wang, 2002).

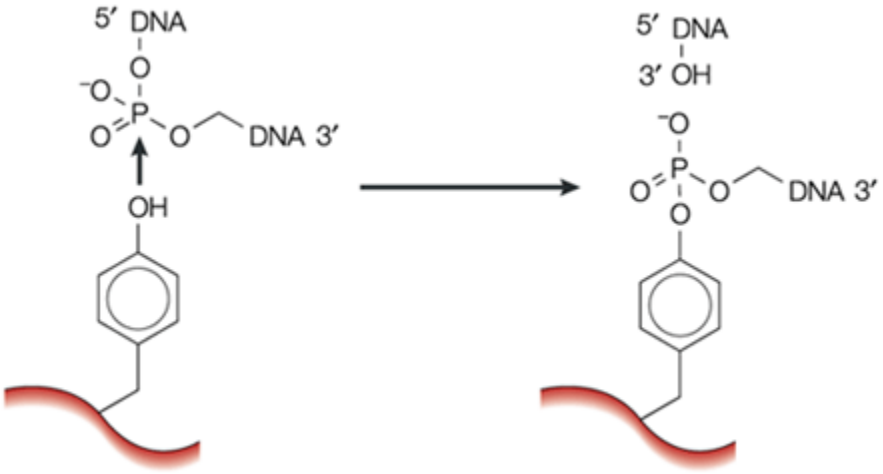


**Figure 5. Catalysis of transient breakage of DNA by DNA topoisomerases.** In the transesterification reaction, the tyrosyl oxygen of the enzyme attacks the DNA phosphorus leading to the breakage of the DNA backbone bond and the formation of a covalent enzyme -DNA intermediate. Rejoining the backbone bond occurs by the reversal of the reaction shown above. In the reverse or second transesterification reaction, the oxygen of the DNA hydroxyl group generated in the first reaction, attacks the phosphorus of the phosphotyrosine link breaking the covalent bond between the protein and DNA and reforms the DNA backbone bond. In the reaction catalyzed by a type IA or type II enzyme, a 3'-OH is the leaving group and the active tyrosyl becomes covalently linked to a 5'-phosphoryl group, as shown. In the reaction catalyzed by a type IB enzyme (not shown) a 5'-OH is the leaving group and the active-site tyrosyl becomes covalently linked to a 3'-phosphoryl group (Wang, 2002). Figure used with permission from Nature Publishing Group.

The end result of this reaction is the creation of an enzyme mediated transient DNA gate through which another DNA strand or a double helix can pass, a phenomenon termed enzyme-bridging mechanism (Wang, 2002). This mechanism is illustrated in Figure 8. This mechanism has been exploited for clinical purposes to develop drugs that act by trapping the covalent enzyme-DNA complex and, this has made these enzymes targets of the pharmaceutical industry, as a lot of quinolone antibiotics (Drlica & Zhao, 1997) and

anticancer drugs (Pommier, 2013; Staker et al., 2005; Staker et al., 2002) wreak havoc by exploiting this route.

### 1.2.1. Classification of topoisomerases

Using DNA strand cleavage as a discriminatory factor, topoisomerases are classified into two broad categories: type I, those that cleave only one DNA strand, and type II, those that cleave both DNA strands to generate staggered double strand breaks (Hartman et al., 2013; Wang, 2002). Further discrimination of the type I topoisomerases in to the subfamily types IA, IB and IC is based on structure and/or mechanistic properties (Hartman et al., 2013). Mechanistic discrimination is based on the point of linkage of the enzyme to the phosphate in the DNA. If the enzyme is linked to a 5' phosphate, they are classified as type IA and if the enzyme is attached to the 3' phosphate, they are classified as types IB (Champoux, 2001; Hartman et al., 2013; Schoeffler & Berger, 2008; Vos et al., 2011; Wang, 2002) and IC (Forterre, 2006; Schoeffler & Berger, 2008; Vos et al., 2011). Prompted by the discovery of a novel type II enzyme from the hyperthermophilic archaeon *Sulfolobus shibatae* (Bergerat et al., 1997; Buhler et al., 1998) the type II topoisomerases were further divided into the subfamilies type IIA and type IIB based on structural differences (Nichols et al., 1999). Type IA topoisomerases are found in all three domains of life namely bacteria, archaea and eukarya (Hartman Chen et al., 2013). The relaxation of negatively supercoiled DNA is their primary activity (Hiasa, DiGate, & Marians, 1994; Wang, 1971) and this requires an exposed single-stranded region within the DNA substrate for activity (Kirkegaard & Wang, 1985). Overwound or positively supercoiled DNA is refractive to type IA enzymes and relaxation of positively supercoiled DNA can only be realized if a pre-existing single-stranded region is present (Kirkegaard & Wang, 1985). Based on the crystal structure of the N-terminal fragment of type IA enzymes, a mechanism of DNA relaxation or catenation-decatenation by type IA topoisomerases has been proposed and confirmed subsequently in biochemical and biophysical reactions (Dekker et al., 2002; Dekker et al., 2003; Li, Mondragon, & DiGate, 2001). In this mechanism, the type IA enzyme, cleaves one strand to generate a single-strand break which is bridged by

the formation of a phosphotyrosine linkage between the enzyme and the 5' end of the broken DNA strand, while holding the 3'-end hydroxyl group non-covalently (Brown & Cozzarelli, 1981; Champoux, 1981; Depew, Liu, & Wang, 1978; Tse, Kirkegaard, & Wang, 1980; Zhang, Cheng, & Tse-Dinh, 2011). Magnesium ions are required for relaxation by type IA topoisomerases, as they are essential for catalysis by helping to keep the 3' end of the cleaved strand in a proper position in the catalytic site (Schmidt et al., 2010; Zhang et al., 2011).

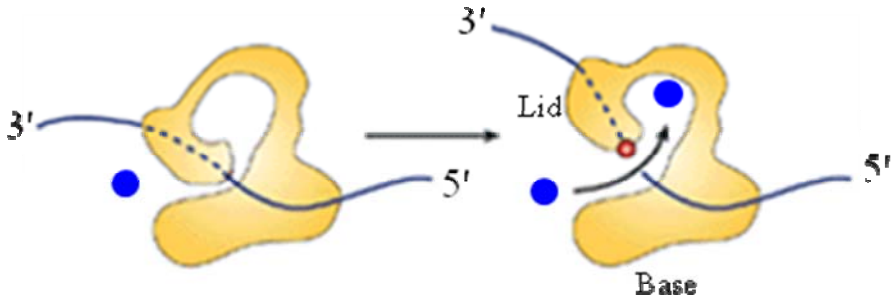


**Figure 6. Mechanism of action of type IA DNA topoisomerases.** Following the transient breakage of a DNA strand ( blue line), the 5' end of the broken DNA is attached covalently to the active-site tyrosyl group (red circle) in the lid of the enzyme, while the 3' end is non covalently bound to the base of the enzyme. The passage of another strand (blue circle) is achieved by lifting the lid away from the base which opens up the gate (Wang, 2002). Figure adapted with permission from *Macmillan publishers Ltd*.

In addition to negative supercoils relaxation, these enzymes can also catalyze knotting, unknotting and interlinking of single stranded circles as well as knotting, unknotting catenation and decatenation of double stranded DNA molecules provided there is a gap in one of them (Dean & Cozzarelli, 1985). Members of this family include bacterial DNA topoisomerase III and I (Srivenugopal, Lockshon, & Morris, 1984; Wang, 1971), eukaryotic DNA topoisomerase III (Wallis et al., 1989) and reverse gyrase (Kikuchi & Asai, 1984). Recent single-molecule techniques have confirmed the general features of the strand passage mechanism in type IA topoisomerases as well as the reduction of linking number in steps of one per catalytic event (Dekker et al., 2002). The energy required to

power reactions catalyzed by type IA topoisomerases is provided by the mechanical tension of supercoiled DNA as reactions catalyzed by type IA topoisomerases proceeds without ATP. One exception is reverse gyrase which uses ATP to introduce positive supercoils and, as its name reflects, its positive supercoiling activity is the opposite of the negative supercoiling activity of DNA gyrase (Kikuchi & Asai, 1984).

Unlike the type IA enzymes which rely on strand passage, type IB enzymes are thought to effectuate supercoil relaxation by swiveling the DNA opposite its nicking point and this mechanism of supercoil relaxation is supported by structural (Stewart et al., 1998) and kinetic (Stivers, Harris, & Mildvan, 1997) data. In this mechanism, a type IB enzyme cleaves a single-strand of the duplex DNA and allows one duplex end to rotate with respect to the other around the intact phosphodiester bond on the opposing strand. In this scheme, only the 3'-OH end of the broken strand is tightly bound to the enzyme through covalent binding with the active site tyrosine residue. Because the 5' end of the DNA strand is only bound to the enzyme via nonspecific interactions, it can rotate freely (Stivers et al., 1997). However, free rotation is hindered by friction between the DNA and the enzyme and this helps to align the broken ends prior to resealing, a mechanism termed "controlled rotation" (Koster et al., 2005).

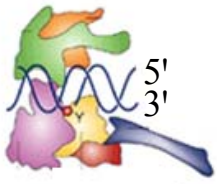


**Figure 7. Mechanism of action of type IB DNA topoisomerases.** In the case of type IB enzymes, the 3' end of the broken DNA is covalently linked to the active-site tyrosyl group (Y) of the enzyme (red circle) (Wang, 2002). Figure used with permission from *Macmillan publishers Ltd*.

The efficiency of relaxation by type IB enzymes is also affected by the extent of supercoiling. It has been shown that higher supercoiling levels lead to an increase in the

mean number of supercoils removed by the enzyme per cleavage/religation cycle, thus indicating that type IB topoisomerases are sensitive to the torque stored in under-or overwound DNA (Koster et al., 2005). The number of supercoils decreases by one per DNA rotation. During one catalytic event, several rotations may occur between the strand cleavage and ligation events, and hence the DNA linking number changes at random by several units, unlike the type IA topoisomerases whereby the linking number changes in steps of one (Koster et al., 2005). Type IB enzymes efficiently relax positively and negatively supercoiled DNA (Madden, Stewart, & Champoux, 1995). The catalytic domains of type IB enzymes and tyrosine recombinases are evolutionarily related (Cheng et al., 1998). Members of this family include eukaryotic DNA topoisomerase I, poxvirus topoisomerase I as well as homologues found in certain bacteria (Krogh & Shuman, 2002) and in *Mimivirus* (Benarroch et al., 2006).

Topoisomerase V (Slesarev et al., 1993) the only known member of the Type IC topoisomerases has so far been found only in the archaea *Methanopyrus kandleri* which is one of the most hyperthermophilic organisms known (Forterre, 2006). Like the type IB enzymes, type IC topoisomerases also relax positively and negatively supercoiled DNA via a nicking and rotation mechanism and requires neither magnesium ions nor ATP as a cofactor (Slesarev et al., 1993; Taneja et al., 2007). Structurally, the active site of type IC enzymes show little similarity to that of type IB and appear to be evolutionarily distinct (Forterre, 2006; Taneja et al., 2006).

As is the case with type IA topoisomerases, type IIA enzymes effect topological changes on DNA by creating an enzyme bridge gap in DNA and passing a second DNA segment through the break (Brown & Cozzarelli, 1979; Mizuuchi et al., 1980). Type IIA topoisomerases however differ in that they cleave both strands of DNA generating a double strand break through which a second duplex is passed (Brown & Cozzarelli, 1981; Liu, Liu, & Alberts, 1980; Mizuuchi et al., 1980; Sander & Hsieh, 1983). To achieve this feat, the enzymes bind and open up a gate in the duplex termed the G segment, and via the transesterification mechanism earlier described, a second DNA piece termed the transfer or T segment is captured and transported through the gate or G segment. Through a second transesterification reaction which is basically the reverse of the first reaction, the gate is

closed and the active site tyrosine is reset for the next catalytic event (Hartman Chen et al., 2013; Wang, 2002). This transient double strand break reaction inverts double-stranded DNA crossovers, changing the LK in steps of two (Brown & Cozzarelli, 1979). Unlike the type IA topoisomerases, type IIA topoisomerases uses ATP as the driving force behind strand passage reactions (Brown & Cozzarelli, 1979; Gellert et al., 1976; Goto & Wang, 1982). This ATP dependent ability to transport one double helix through another equally endows these topoisomerases with the ability to resolve catenanes, the decatenation of double stranded rings, as well as the relaxation of positively or negatively supercoiled DNA (Champoux, 2001; Mizuuchi et al., 1980; Wang, 1996, 1998).

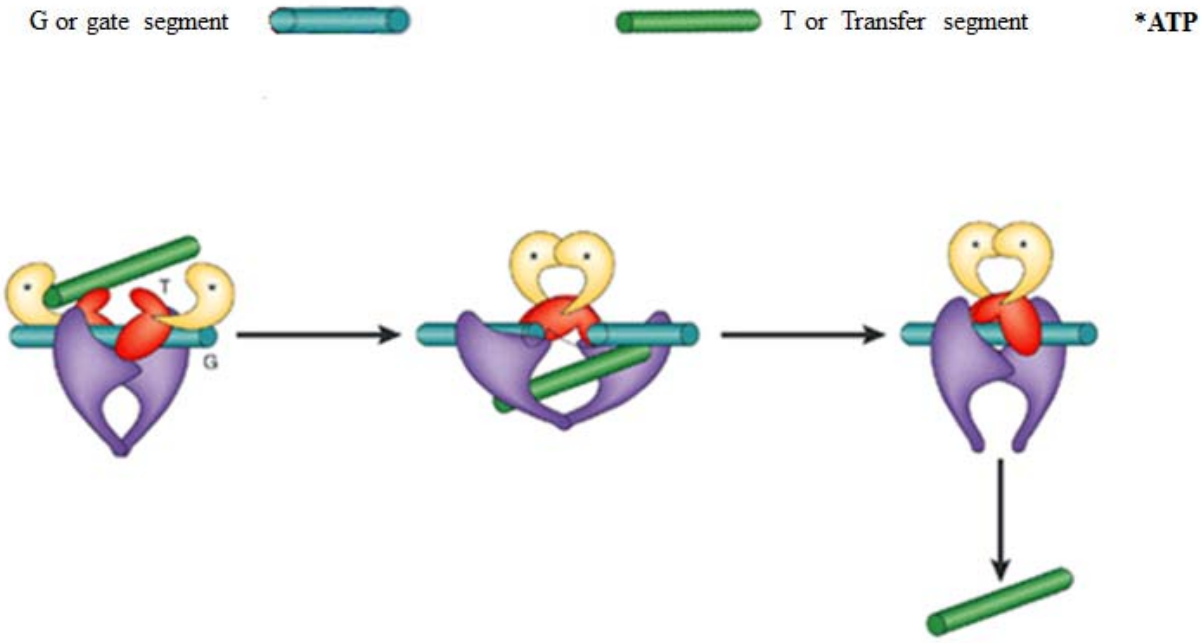


**Figure 8. Mechanism of the two gate model of type II DNA topoisomerases**. The G segment represented by the blue rod is the double stranded DNA segment that contains the enzyme-mediated DNA gate through which the T or transfer segment represented by the green rod is passed through. The transport and subsequent exit of the T-segment through the gate is mediated by ATP binding and hydrolysis respectively. ATP binding sites are represented by asterisks (Wang, 2002). Figure adapted with permission from *Macmillan publishers Ltd*.

Type IIA topoisomerases are widely distributed in all cellular organisms and they include: eukaryotic topo II (Baldi et al., 1980; Hsieh & Brutlag, 1980; Miller, Liu, & Englund, 1981), viral and bacteriophage topo II (Lavrukhin et al., 2000; Liu, Liu, & Alberts, 1979; Raoult et al., 2004; Stetler, King, & Huang, 1979), bacterial, chloroplast, and archael DNA gyrase (Gellert et al., 1976; Sioud et al., 1988; Thompson & Mosig, 1985), and bacterial topo IV (Kato et al., 1990). Bacterial DNA gyrase is the only known type II A topoisomerase that is capable of introducing negative supercoils into DNA (Gellert et al., 1976).

Type IIB topoisomerases like their type IIA counterparts, can relax both positive and negative supercoils by strand passage utilizing ATP in the process (Bergerat, Gadelle, & Forterre, 1994). Type IIB topoisomerases are found in archaea, plants, and some bacterial, protists and algal lineages (Bergerat et al., 1997; Malik et al., 2007). So far this family is represented by topo VI (Bergerat et al., 1997).

In *E. coli* four topoisomerases have been identified with two belonging to the type I A family (topoisomerase I and topoisomerase III) and two belonging to the type IIA family (DNA gyrase and topoisomerase IV).

### 1.2.2. Topoisomerase I (topo I)

This was the first topoisomerase to be identified (Wang, 1971). It is encoded by the gene *topA* located in the *cys-trp* region of the bacterial chromosome (Sternglanz et al., 1981; Trucksis & Depew, 1981). The first function attributed to this topoisomerase is the relaxation of negative supercoils (Wang, 1971) achievable only in the presence of an exposed single-stranded DNA region (Kirkegaard & Wang, 1985). The preference of topo I for negatively supercoiled DNA is therefore rooted in its selective binding to single-stranded DNA since negative supercoiling is important for processes that require strand separation such as transcription and DNA replication. Proof of this came through biochemical experiments demonstrating that positively supercoiled DNA can be relaxed to the same efficiency by topo I provided it has a single-strand loop (Kirkegaard & Wang, 1985). One function of topo I is regulating supercoiling homeostasis along with gyrase (Zechiedrich et al., 2000). Studies in *E. coli* have highlighted a global supercoiling regulation system maintained by the opposing enzymatic actions of topo I and gyrase with topo I relaxing negative supercoils while gyrase (to be discussed below) introduces negative supercoils to ensure balanced growth (Drlica, 1992). Even though some studies have shown that *E. coli topA* is not essential for viability (Stupina & Wang, 2005), previous work on *E. coli* has shown that *topA* mutants can acquire compensatory mutations during growth that reduce negative supercoiling, and these mutations are located in gyrase genes (DiNardo et al., 1982; Pruss, Manes, & Drlica, 1982). One of the most

crucial roles of topo I is its involvement in preventing the formation of DNA/RNA hybrids (R-loops) during transcription by relaxing transcription-induced negative supercoils generated by the movement of RNA polymerase along the DNA template (Drolet et al., 1994). In fact plasmids extracted from bacteria with mutations inactivating *topA* are hypernegatively supercoiled and this excess supercoiling depends on transcriptional activity (Masse & Drolet, 1999a; Pruss, 1985; Pruss & Drlica, 1986; H. Y. Wu, Shyy, Wang, & Liu, 1988).

In an *in vitro* study demonstrating the role of topo I in R-loop prevention, it was found that in the presence of DNA gyrase and in the absence of topo I, transcription elongation caused the template DNA to be hypernegatively supercoiled (DNA is said to be hypernegatively supercoiled if it cannot be resolved by DNA intercalators in an agarose gel) and this was linked to the formation of R-loops (Drolet et al., 1994; Phoenix et al., 1997). It has equally been found in *in vitro* studies that the addition of RNase HI (encoded by *rnhA*), an enzyme that degrades the RNA moiety of an R-loop, inhibits the formation of hypernegatively supercoiled DNA (Phoenix et al., 1997). The *in vivo* significance of R-loops was confirmed when it was found that in the presence of active gyrase, overproducing RNase HI corrected the growth defects of *topA* null mutants (Drolet et al., 1995). Interestingly, it has been found that *topA rnhA* mutants exhibit growth and chromosome segregation defects (Stockum, Lloyd, & Rudolph, 2012; Usongo et al., 2008). It has equally been found that the growth defect of various *topA* null mutants correlates with the formation of R-loop dependent hypernegatively supercoiled DNA implying that one major role of topo I is to relax transcription-induced negative supercoiling (Massé & Drolet, 1999a, 1999b). More recently, studies in our laboratory have shown that excess negative supercoiling inhibits growth by causing RNA degradation which in turn affects protein synthesis (Baaklini et al., 2008; Drolet, 2006).

The role of topo I in removing transcription-driven supercoiling is supported with biochemical evidence showing its physical interaction with RNA polymerase. In one study, the lethality of Tn*5* transposase overproduction in *E. coli* was linked to titration of topo I (Yigit & Reznikoff, 1999). It was found that the lethal derivatives of Tn*5* transposase co-purified with topo I while the non-lethal derivatives did not. Incidentally, RNA polymerase

was also found to co-purify with Tn*5* but the co-purification was reduced in extracts of a *topA* mutant strain implying that the interaction between RNA polymerase and topo I was responsible for the co-purification of RNA polymerase with Tn*5* transposase (Yigit & Reznikoff, 1999). This indirect evidence was a proof showing the interaction between topo I and RNA polymerase. More direct evidence showing the interaction between topo I and the RNA polymerase complex has been demonstrated. It has been shown that protein-protein interactions link the β' subunit of RNA polymerase and the C-terminal domains of topo I, which are homologous to the zinc ribbon domains in a number of transcription factors (Cheng et al., 2003).

Another dominant role of topo I is the prevention of thermal denaturation bubbles. It has been observed that mutations in *topA* lead to increased thermosensitivity (Qi, Menzel, & Tse-Dinh, 1999). During heat shock, the rapid induction of heat shock genes may lead to localized accumulation of negative supercoils at these gene loci which must be rapidly relaxed by topo I to prevent the formation of R-loops. Interestingly it has been found that *topA* mutants are readily killed by various stresses (Qi, Menzel, & Tse-Dinh, 1996; Rui & Tse-Dinh, 2003; Tse-Dinh, 2000). In one study, it was found that RNase HI overexpression in *topA* mutants partially restored the $\sigma^{32}$ dependent induction of stress induced genes in response to high temperature and ethanol, and also improved the survival rate of *topA* mutants following high temperature and oxidative challenges (Cheng et al., 2003). Judging from the sensitivity of *topA* mutants from various stresses, it is not surprising that the synthesis of topo I is directed by four promoters of which two, P1 and Px1 are respectively under the control of σ32 involved in the heat-shock response and σS implicated with the general stress response (Rui & Tse-Dinh, 2003).

### 1.2.3. Topoisomerase II (DNA gyrase)

DNA gyrase was discovered in 1976 (Gellert et al., 1976) as the only topoisomerase that introduces negative supercoils into relaxed or positively supercoiled DNA in the presence of ATP. Gyrase is a heterotetramer composed of two A and two B subunits ($GyrA_2GyrB_2$) (Higgins et al., 1978; Klevan & Wang, 1980). Gyrase is capable of introducing negative supercoils into DNA due to its ability to wrap DNA around the C-

terminal domain (CTD) of its A-subunit (GyrA) forming a positive supercoil, a phenomenon described as chiral wrapping (Kampranis & Maxwell, 1996; Liu & Wang, 1978a, 1978b; Reece & Maxwell, 1991). Following mutational studies, a sequence of 7-amino acids that is essential for chiral wrapping of DNA around gyrase has been identified in the GyrA box (Kramlinger & Hiasa, 2006). To carry-out strand passage, a 140-base pair of DNA segment is wrapped around the enzyme. This wrapping allows the G-(gate segment) and T-(transfer segment) to be closely spaced on the same DNA, thus favoring intramolecular strand passage with the appropriate orientation for unidirectional strand passage (Wang, 2002). Upon transfer of the T-segment across the G-segment, the positive supercoil is converted into a negative supercoil by reducing the linker number in steps of two, in accordance with the sign inversion model (Brown & Cozzarelli, 1979). The unique ability of gyrase to introduce negative supercoiling depends crucially on the C-terminal domain of its A-subunit. Studies have shown that deleting the C-terminal domain of gyrase simply converts gyrase into a conventional (DNA-relaxing) type II topoisomerase, also implying that the unique properties of gyrase are attributable to DNA wrapping (Kampranis & Maxwell, 1996). The importance of DNA wrapping in the negative supercoiling reaction of gyrase has also been demonstrated by single-molecule studies. In these studies, it has been shown that at high DNA tensions, gyrase loses its ability to wrap DNA and its ability to introduce supercoils, but not its ability to relax positive supercoils (Nollmann et al., 2007). Other studies with single molecules have equally shown that DNA tension affects the processivity of gyrase, with greater tensions increasing the probability that the enzyme will pause and decreasing the probability that it will initiate another round of supercoiling (Gore et al., 2006). All these studies do confirm that DNA wrapping is essential for gyrase supercoiling function.

The N-terminal domain of GyrB is responsible for binding and hydrolysis of ATP (Mizuuchi, O'Dea, & Gellert, 1978; Sugino et al., 1978). This domain forms the entrance gate (N gate) for the T segment. Opening and closure of this gate is mediated by ATP binding and hydrolysis (Roca & Wang, 1992). Binding of ATP to gyrase drives the supercoiling reaction while hydrolysis of ATP to ADP serves to reset the enzyme for a second round of catalysis. In the absence of ATP, gyrase has been shown to relax negative

supercoils (Nollmann et al., 2007). By determining the final level of supercoiling that can be achieved, the ATP/ADP ratio plays a major role in the supercoiling-relaxation relationship (Drlica, 1992). This makes gyrase and supercoiling sensitive to intracellular energetics, which are themselves sensitive to extracellular environmental factors such as salt concentration, oxygen tension, temperature and pH (Cameron, Stoebel, & Dorman, 2011).

The major *in vivo* function of gyrase as stated above is the introduction of negative supercoils into the chromosome. Proof of this major role is the observation that the chromosome becomes relaxed if gyrase is inhibited (Drlica & Snyder, 1978; Lockshon & Morris, 1983; Steck et al., 1984). In fact initial measurements of the superhelical density of the *E. coli* chromosome shows that the chromosome is maintained at a physiological level of *ca*.-0.05 (Sinden et al., 1980) by the combined action of gyrase, topo I and topo IV with the latter two acting as a counter balance to gyrase activity by relaxing negative supercoils (Zechiedrich et al., 2000). As we shall see ahead, the role played by gyrase in maintaining global supercoiling is critical for another important role of gyrase in the cell: DNA replication. DNA gyrase can also help to bend and fold DNA. This function of gyrase has been observed in bacteriophage Mu which contains a strong gyrase binding site. Inactivating this site blocks replication by constraining the prophage ends in the host genome for both ends need to be brought together prior to replication (Oram et al., 2006; Pato, 1994; Pato & Banerjee, 1999; Pato, Howe, & Higgins, 1990; Pato et al., 1995). The suppression of a growth defect due the absence of HU, a small DNA-bending protein by gyrase mutations is also likely related to the ability of gyrase to bend DNA (Malik et al., 1996).

### 1.2.4. Topoisomerase III (topo III)

This topoisomerase is encoded by the gene *topB* (DiGate & Marians, 1989). In *E. coli* most of the functions attributed to topo III have been shown through *in vitro* experiments. *In vitro*, its decatenation activity, which is one of its most interesting activities, has been clearly demonstrated. *E. coli* cell extracts containing topo III have been shown, in an *in vitro* assay, to efficiently decatenate pBR322 replication intermediates

and catenated DNA products with single-stranded gaps and nicks (DiGate & Marians, 1988), a function that is attributed to the presence of a decatenating loop sequence in the enzyme (Mondragon et al., 2000). In addition, it has been shown in a reconstituted replication assay *in vitro* that, by acting on single stranded DNA at the replication fork, topo III alone can support replication by removing precatenanes (Hiasa & Marians, 1994a; Nurse et al., 2003). Other studies have equally questioned the role of topo III in DNA unlinking. In one *E. coli* study in the absence of topo IV, nearly all the synthesized plasmid DNA was catenated (Zechiedrich & Cozzarelli, 1995). Compared to the effects caused by the loss of only topo IV which is the major cellular decatenase, additional removal of topo III does not cause larger accumulation of catenated intermediates during DNA replication (Lopez et al., 2005). When overproduced, topo III may physically remove precatenanes (Hiasa et al., 1994; Hiasa & Marians, 1994a; Nurse et al., 2003). However, the *in vivo* significance of this reaction is questionable as the enzyme needs to be overproduced to perform this function.

Irrespective of these nuances, more recent results have attributed an *in vivo* function for this topoisomerase. The suppression of the Par (formation of large unsegregated nucleoid in midcell) and growth defects of temperature sensitive (Ts) mutants of *parE* and *parC* (genes encoding the subunit of topo IV) at restrictive temperatures by high copy suppressors depends strictly on *topB* (Perez-Cheeks et al., 2012). More recently we have shown that both the growth defect and the Par$^-$ phenotypes of a *gyrB*(Ts) mutant at nonpermissive temperature are corrected by deleting *topA* but only when *topB* is present (Usongo et al., 2013). Therefore, topo III appears to play a role during replication to allow chromosome segregation.

Some studies have suggested functional interactions between topo I and III. In one study, topo III was isolated as a multicopy suppressor of *topA* (Broccoli, Phoenix, & Drolet, 2000). Double *topA topB* mutants were shown to be very sick (Stupina & Wang, 2005; Usongo et al., 2013; Zhu, Pongpech, & DiGate, 2001). Cells harvested from these mutants exhibit extensive filamentation and unsegregated nucleoids (Stupina & Wang, 2005; Usongo et al., 2013; Zhu et al., 2001). Interestingly a mutation in the key recombination gene *recA* corrects the phenotypes of these double mutants suggesting that

recombination intermediates not resolved by these enzymes are toxic to the cell (Zhu et al., 2001). In *S. cerevisiae,* loss of topo III (TOP3) results in increase recombination between short repeated sequences. These observations seem to suggest that defects in type 1A topo activity can affect chromosomal stability due to the failure to resolve recombination intermediates.

A universal family of proteins involved in DNA repair is the RecQ helicase family (Bernstein, Gangloff, & Rothstein, 2010; Cheok et al., 2005; Chu & Hickson, 2009; Laursen et al., 2003). It has been suggested that interactions between RecQ-like helicases and topo III may prevent the accumulation of lethal recombination intermediates (Lopez et al., 2005). Interestingly, it has been demonstrated *in vitro* that RecQ helicase and single stranded binding protein (SSB) can act in concert with topo III in the resolution of converging replication forks (Suski & Marians, 2008). The role of RecQ in this reaction is to provide the single stranded DNA region needed for the binding of topo III, and SSB mediates the functional interaction between RecQ and topo III. However, Marians *et al*., have been unable to demonstrate a functional interaction between RecQ and topo III *in vivo* (Perez-Cheeks et al., 2012). RecQ helicase has also been shown *in vitro* to stimulate topo III to fully catenate double-stranded DNA (Harmon, DiGate, & Kowalczykowski, 1999). The association between a helicase and topo III has also been reported in eukaryotes. In yeast, interaction between topo III and Sgs1 (*E. coli* RecQ homolog), has been proven as well as topo III involvement in the recombination pathway (Ui et al., 2005). Five RecQ-like helicases have also been identified in humans and they include RECQ1, BLM, WRN, RECQ4 and RECQ5 (Bernstein et al., 2010; Chu & Hickson, 2009). Of these five, inactivation of three cause the rare genetic disorders Bloom syndrome (BLM), Werner syndrome (WRN) and Rosthmund-Thomson syndrome (RECQ4) which carry serious health consequences including premature aging and increase risk to cancer (Chu & Hickson, 2009). A physical and functional relationship has also been established between human Top3α and BLM (L. Wu et al., 2000). As we shall see ahead, this interaction between a helicase and topoisomerase III plays a major role in the resolution of Holliday junction intermediates following homologous recombination. The pathways involving a helicase and topo III in recombination in eukaryotes is well established (Hartman Chen et

al., 2013). In *E. coli*, whether type 1A topos act with RecQ is still debated (Lopez et al., 2005; Perez-Cheeks et al., 2012). Irrespective of all these studies suggesting possible roles for topo III, in *E. coli*, its main role is in decatenation and this is only possible if single-stranded regions are present on the DNA (Digate & Marians, 1988).

### 1.2.5 Topoisomerase IV (topo IV)

Like DNA gyrase, topo IV is a heterotetramer $(Top4C)_2(Top4E)_2$ made up of two ParC and two ParE subunits that are homologous to GyrA and GyrB respectively (Kato et al., 1990; Kato, Suzuki, & Ikeda, 1992; Peng & Marians, 1993). Being a type II topoisomerase like gyrase, topo IV uses a double-strand passage mechanism (Hartman Chen et al., 2013; Wang, 2002). Irrespective of the fact that both enzymes are sensitive to the same inhibitors, the coumarins and quinolone families of antibiotics (Peng & Marians, 1993), they are functionally different since topo IV does not introduce negative supercoils (Ullsperger & Cozzarelli, 1996). This functional difference is rooted in the C-terminal domains of these enzymes (Corbett et al., 2005; Hsieh et al., 2004). Though like the GyrA subunit of gyrase, the CTD of the ParC subunit of topo IV can bind DNA, unlike GyrA, it cannot wrap DNA around itself (Corbett et al., 2005; Peng & Marians, 1995). The importance of wrapping as a discriminatory factor in the functional differences of these two enzymes is confirmed by the observation that deleting the CTD of the A subunit of gyrase simply converts gyrase into an enzyme with a strong decatenating activity much like that of topo IV (Kampranis & Maxwell, 1996). In fact, the absence of the GyrA box in topo IV CTD prompted the CTD of topo IV to be described as the "broken form" of gyrase CTD (Hsieh et al., 2004) or as a "degenerate form" homologous to GyrA CTD (Corbett et al., 2005).

DNase I footprinting and exonuclease digestion experiments have deciphered the mode of gyrase and topo IV binding to DNA. Through these studies, it has been revealed that a region of about 140 base pairs is protected following binding by gyrase and this allows the G and T segments to be close to each other for processing (Fisher et al., 1981; Kirkegaard & Wang, 1981; Liu & Wang, 1978a; Orphanides & Maxwell, 1994) while topo IV binding protects a region of about 34 base pairs and it bends DNA to an extent that is

not sufficient to generate a T segment flanking a G segment (Corbett et al., 2005; Peng & Marians, 1995). It is in this light that wrapping by gyrase favors intra rather than intermolecular strand passage while wrapping by topo IV favors the contrary. This gives gyrase a poor decatenating activity relative to its supercoiling and relaxing activities and explains the inability of topo IV to introduce negative supercoils. It also explains why its decatenating activity is better than its relaxation activity.

Even though *in vitro* topo IV has been shown to be able to catalyze the relaxation of supercoiled DNA (Kato et al., 1992), recent studies have shown that topo IV relaxes positive supercoils much faster than negative supercoils (Charvin et al., 2003; Crisona et al., 2000; Stone et al., 2003). However, it has also been shown that both positive and negative supercoils compete equally well with linear DNA for topo IV binding (Stone et al., 2003). Single molecules studies on braided DNA molecules have shown that topo IV actually senses DNA crossings rather than supercoiling per se. It has been established that topo IV preferentially acts on left handed braids having local segment juxtaposition geometry similar to that of positively supercoiled (Charvin et al., 2003; Crisona et al., 2000; Stone et al., 2003). A recent study demonstrates that topo IV acts processively on positively supercoiled DNA, whereas its activity on negatively supercoiled DNA is distributive (Neuman et al., 2009). This may explain why topo IV only plays a secondary role in negative supercoiling removal (Zechiedrich et al., 2000). Topo IV plays a major role in chromosome segregation at the end of DNA replication (Ullsperger & Cozzarelli, 1996; Wang et al., 2008; Zechiedrich & Cozzarelli, 1995). Interestingly, topo IV has been shown to interact with FtsK and the chromosomal *dif* sites, both involved in the resolution of chromosomal dimers by the XerCD proteins (Espeli et al., 2003; Hojgaard et al., 1999; Wang, West & Shapiro, 2006) and also with the cellular condensin MukB that is responsible for chromosome compaction to enable efficient chromosome segregation (Hayama et al., 2013; Li et al., 2010).

## 1.3. DNA replication

Bacteria must duplicate their genomes in order to enable cellular proliferation and moreover, genome duplication must occur at the correct time, and only once, during the cell

cycle (Katayama et al., 2010; Skarstad, Boye, & Steen, 1986). The first step in the events leading to genome duplication is initiation. In *E. coli*, this requires the binding of the initiator protein DnaA to the single origin of replication *oriC* (Messer, 2002). This is a very accurate event and in fact the precision in timing of initiation is very critical especially for bacteria growing in rich media where new rounds of replication are initiated before the completion of previous rounds, and where newly divided daughters cells inherit chromosomes with active replication forks and multiple origins (Cooper & Helmstetter, 1968; Skarstad et al., 1986). It has been shown that all copies of *oriC* in these cells initiate DNA replication synchronously. This implies that there is an effective mechanism in place to achieve this feat (Skarstad et al., 1986). The sequential events that ultimately culminate in the pre-replicative complex assembly and the loading of the DnaB helicase begin with the unwinding of the duplex DNA within the *oriC* region by DnaA. This defines the origin and set the stage for the pre-RCs assembly (Leonard & Grimwade, 2005; Prasanth et al., 2004).

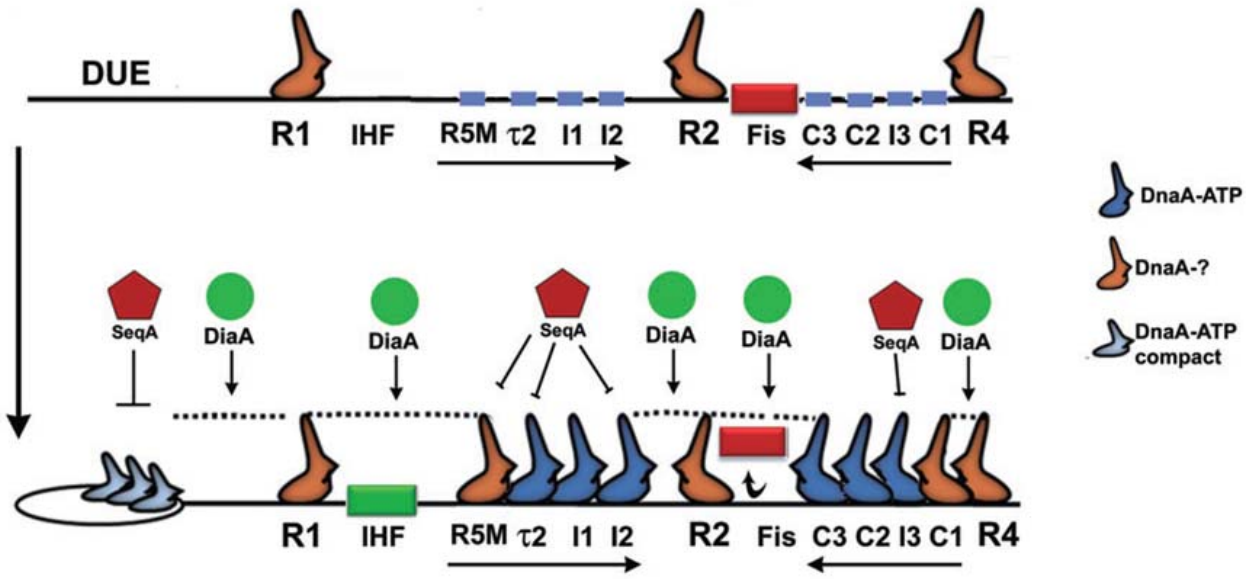


**Figure 9. A revised map of *oriC* depicting the model of pre-RC assembly.** In the first stage of pre-RC assembly, DnaA binds to the three high affinity sites R1 R2 and R4 and Fis also binds. In the second stage of pre-RC assembly, the high affinity sites help DnaA to bind to the low affinity sites (marked in blue) and DnaA must be coupled to ATP to bind these sites. Extension of DnaA oligomers displaces Fis (red rectangle), and IHF (green rectangle) binds in this stage, modulating the distance between the strong and weak sites. SeqA the negative regulator (red pentagon) prevents oligomer extension and unwinding by blocking sites containing GATC. The positive regulator DiaA (green circle) is proposed to stabilize DnaA interactions that are required to extend DnaA from the nucleation sites, and to connect the two converging oligomers at R2. Following the filling and joining of arrays, a compact helical filament of DnaA-ATP is formed that extend from R1 into the DUE (DNA unwinding element) and DiaA may also stabilize this extension (Rozgaja et al., 2011). Figure used with permission from John Wiley and Sons.

Sequence analysis of the *oriC* region has revealed that *oriC* has an array of nine base pair sequence motifs termed DnaA boxes that are bound by DnaA (Fuller, Funnell, & Kornberg, 1984). DnaA binds with highest affinity to the DnaA box consensus sequence 5'-TTATCCACA-3' (Schaper & Messer, 1995) also termed the R-box (Zyskind et al., 1983). These DnaA binding sites include the high affinity sites R1, R2 and R4 (Margulies & Kaguni, 1996; Schaper & Messer, 1995) and the low-affinity sites, R3, R5, I1, I2, I3, τ1, τ2 (Kawakami, Keyamura, & Katayama, 2005; Leonard & Grimwade, 2011; Margulies & Kaguni, 1996; McGarry et al., 2004; Ozaki & Katayama, 2012; Rozgaja et al., 2011), C3, C2 and C1 (Rozgaja et al., 2011). Even though these sites are important for *oriC* function, it has been found that only the left half of *oriC* is required for viability under slow growth conditions and both sides are required under conditions of rapid growth (Stepankiw et al., 2009). Binding sites in *oriC* are also recognized by the SeqA protein (Slater et al., 1995), DiaA (Ishida et al., 2004), IHF and Fis (Roth, Urmoneit, & Messer, 1994). These proteins modulate DnaA activity and by doing so they affect initiation (Leonard & Grimwade, 2005). In addition to the DnaA binding sites, *oriC* equally carries near its left border, an AT-rich duplex unwinding element (DUE) made up of three 13-mer motifs that become unwound following binding by DnaA (Bramhill & Kornberg, 1988).

### 1.3.1. The replication initiator DnaA

DnaA is a member of $AAA^+$ family of proteins conserved in all domains of life (Duderstadt & Berger, 2008; Katayama et al., 2010). The activity of DnaA is regulated by its binding to ATP (Sekimizu, Bramhill, & Kornberg, 1987). Studies have shown that there are about 1000-2000 monomers of DnaA per cell in *E. coli* (Sekimizu et al., 1988) though it has been reported that only about 20 DnaA monomers are needed to form a pre-replicative complex (Crooke et al., 1993; Ryan et al., 2004) implying that there is more DnaA available than required. Irrespective of its cellular abundance, DnaA still sets the cell mass at initiation (Lobner-Olesen et al., 1989). Biochemical and structural studies combined with genetic approaches have revealed that DnaA contains four distinct domains (Kaguni, 2006; Katayama et al., 2010). Domain I (the-N-terminal, amino acids 1-90) is used primarily for protein-protein interactions. This domain equally interacts with the DNA

replicative helicase (DnaB) (Abe et al., 2007) as well as with DiaA (Ishida et al., 2004). This domain also acts in DnaA oligomerization (Simmons et al., 2003). Domain II amino acids (90-130) is the only non-conserved region of DnaA and functions as a flexible linker whose length varies among DnaA homologs (Abe et al., 2007; Molt et al., 2009). This domain does not yet have any specific function as studies have shown that systemic mutations can be introduced into this region without any loss of cell viability (Messer et al., 1999; Nozaki & Ogawa, 2008). Domain III amino acids (130-347) contains the sensors I, II (box VIII) and box VII motifs of the AAA+ protein family (Erzberger & Berger, 2006). It functions in the binding and hydrolysis of ATP (Erzberger et al., 2002). Domain IV the C-terminal domain amino acids (347-467) contains a helix-turn-helix motif for double stranded DNA binding (Erzberger et al., 2002; Fujikawa et al., 2003) and a region for membrane interaction that serves to regulate replication initiation (Garner & Crooke, 1996). Domain IV also recognizes and binds to the DnaA box motif (Blaesing et al., 2000; Erzberger et al., 2002; Fujikawa et al., 2003; Roth & Messer, 1995; Sutton & Kaguni, 1997).

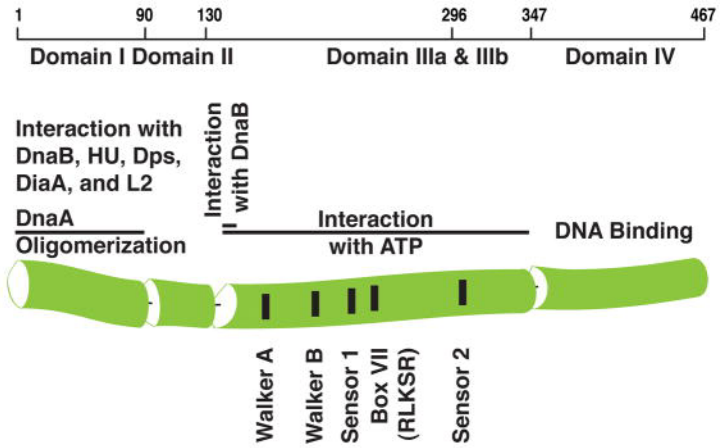


**Figure 10. Domain organization of DnaA**. A region near the N-terminus is involved in the interaction between DnaA and DnaB, HU, Dps, DiaA. This region is also required for DnaA oligomerization. Domain II functions as a flexible linker connecting domain I, IIIa and IIIb. Domain III is involved in ATP binding while Domain IV is involved in DNA binding (Kaguni, 2011). Figure used with permission from Elsevier (Kaguni, 2011).

To begin initiation, DnaA first binds to the high affinity sites at *oriC* (Miller et al., 2009). Domain IV of DnaA is required for efficient localization to these sites. Biochemical and genetic studies reveal that two specific elements of Domain IV, which are the DnaA signature sequence and a region known as the basic loop, are required for efficient localization at *oriC* (Blaesing et al., 2000; Sutton & Kaguni, 1997*)*. The DnaA signature sequence mediates specific recognition of the DnaA box binding site while the basic loop contains a conserved arginine residue that is crucial for DNA binding (Blaesing et al., 2000; Sutton & Kaguni, 1997). These two motifs thus define the high-affinity interactions between DnaA and its cognate binding sites at *oriC*. The high affinity sites define the origin and these sites form the structural platform that is used to assemble the pre-replicative complex. Binding of DnaA to the high-affinity sites helps this protein to bind to the lower affinity sites. In fact, high affinity sites play a critical role in pre-replicative complex assembly as it has been shown that DnaA cannot occupy any low affinity recognition sites in *oriC* without the cooperative assistance from DnaA bound to the high affinity sites (Miller et al., 2009). It has been reported that the loss of high affinity sites have a

detrimental effect on *oriC* function (Langer et al., 1996). More importantly, it has been shown that loss of the high affinity site R1 results in a nonfunctional *oriC* (Weigel et al., 2001) while the loss of the high affinity sites R2 and R4 results in defective initiation timing (Weigel et al., 2001) and loss of R4 alone also results in severe perturbation in the timing of initiation (Riber et al., 2009).

DnaA occupation of low affinity sites via cooperation from the high affinity sites occur only in the presence of ATP (McGarry et al., 2004). DNA footprinting studies using dimethyl sulfate shows that five DnaA binding sites (R-boxes) bind both active ATP-DnaA and inactive ADP-DnaA with equal affinity. This is not the case for the low affinity I sites which bind DnaA only in the presence of ATP (McGarry et al., 2004). In fact, it has been found that single base substitutions in either I2 or I3 results in reduced amounts of bound ATP-DnaA *in vitro* and inactivate replication of *oriC*-containing plasmids *in vivo* (McGarry et al., 2004). Binding of DnaA-ATP to the lower affinity 9 mer sites (R5M, I sites, and τ sites) that are dispersed on each half of *oriC* leads to DNA strand separation in the AT rich DNA unwinding element (DUE) (Bramhill & Kornberg, 1988). The interplay between two distinct modes of DNA binding by DnaA underlie the remodeling activity of DnaA at *oriC* (Duderstadt et al., 2010) whereby the high-affinity interaction with strong DnaA boxes localizes DnaA to the origin, while the ATP-dependent and cooperative interactions with weaker affinity sites allow DnaA to form an oligomeric complex that is able to melt the AT-rich DUE (Duderstadt et al., 2010).

Recent studies have also shown that nucleoid associated proteins influence the interactions between DnaA and *oriC* in assembling the pre-replicative complex. IHF binds to the left half between R1 and R5M and bends *oriC* (Roth et al., 1994) and loss of IHF though not important for viability, results in perturbations in initiation timing, implying that IHF plays a role in modulating the binding of DnaA in pre-replicative complex assembly (Roth et al., 1994). The protein DiaA which binds DnaA domain I have been shown to help in the process of pre-replicative complex assembly by stimulating the formation of ATP-DnaA multimers on *oriC* (Keyamura et al., 2009). Studies have also shown that strains lacking *diaA* have untimely initiations (Ishida et al., 2004). HU also influences the

unwinding of *oriC* by DnaA as strains lacking *hupA* that encodes the alpha subunit of HU have untimely initiations (Bahloul et al., 2001).

### 1.3.2. DnaB

The separation of the two strands of DNA is carried out by the replicative helicase DnaB and its action is required for the movement of the replication fork. This protein has six identical protomers that assemble into a ring-like structure that serves as a passage for single stranded DNA template during replication (Lo et al., 2009). Studies in *E. coli* have shown that this protein unwinds DNA by translocating in the 5'→3' direction on the single strand DNA to which it is bound (Lee et al., 1989; Richardson & Nossal, 1989). It has equally been shown that DnaB must be in complex with its partner DnaC which regulates the helicase function of DnaB in order for it to be delivered at *oriC* to initiate DNA replication (Davey et al., 2002; Mott et al., 2008). Structural studies have revealed that DnaB protomer has a large C-terminal domain that contains a RecA-like fold and a smaller N-terminal domain (Bailey, Eliason, & Steitz, 2007). DnaC binds to the larger C-terminal domain with three DnaC monomers for each DnaB hexamer (Makowska-Grzyska & Kaguni, 2010). The N-terminal region of DnaC is necessary for its interaction with DnaB (Ludlam et al., 2001).

### 1.3.3. DnaC

As mentioned above, the molecular escort protein DnaC is the essential partner for DnaB for its delivery at *oriC*. It is a member of the $AAA^+$ family of ATPases that contain specific amino acid sequence motifs that serves for ATP binding and hydrolysis (Koonin, 1993). Even though members of the $AAA^+$ family of proteins have a high affinity for ATP, DnaC binds weakly to ATP with a dissociation constant ($K_d$) of about 8μM (Biswas, Flowers, & Biswas-Fiss, 2004; Davey et al., 2002) and is also a weak ATPase itself (Davey et al., 2002; Wahle, Lasken, & Kornberg, 1989). The influence of ATP in DnaC function has been a puzzle but however, new evidence now suggests that the activation of its ATPase only occurs in an early step in *oriC* replication with maximal activity obtained in the presence of both DnaB and ssDNA (Davey et al., 2002). ATP stimulates ssDNA

binding by DnaB-DnaC complex and expands the ssDNA bubble at the origin (Davey et al., 2002). Studies have also shown that DnaC interacts with DnaA, and this interaction suggests that DnaC collaborates with DnaA to load the DnaB helicase (Mott et al., 2008). The ATPase and helicase activities of DnaB are inhibited when it is in complex with DnaC, so DnaC must dissociate from DnaB in order for helicase activation to occur (Davey et al., 2002; Mott et al., 2008). In fact, studies have shown that mutant DnaCs that are defective in ATP binding due to amino acid substitutions in the Walker A box, a nucleotide binding motif, do not inhibit DnaB *in vivo* and fail to deliver DnaB to *oriC in vitro* (Davey et al., 2002; Ludlam et al., 2001). This supports the ATP/ADP switch hypothesis whereby DnaC possesses dual states, each with its own positive role in replication. DnaC-ATP state inhibits DNA unwinding by DnaB though this state is required to load DnaB onto *oriC*, and the DnaC-ADP state that does not inhibit DnaB but decreases the grip of DnaC on ssDNA (Davey et al., 2002).

### 1.3.4. Helicase loading by DnaA

Following unwinding of *oriC*, ATP activated DnaA loads the DnaB-DnaC complex onto each of the separated strand of *oriC* with one DnaB-DnaC complex loaded on the top strand near the left border of *oriC* and a second DnaB-DnaC complex loaded on the lower strand next to the DnaA box R1 (Carr & Kaguni, 2001; Fang et al., 1999). Several studies support the conclusion that DnaA loads DnaB-DnaC at *oriC.* Firstly, ATP or ATPγS bound to DnaC does not affect the DnaA-dependent loading of DnaB-DnaC complex at *oriC* (Davey et al., 2002; Makowska-Grzyska & Kaguni, 2010). The fact that DnaC still remains bound to DnaB in the presence of either ATP or its analog suggests that the act of helicase loading does not stimulate the hydrolysis of ATP bound to DnaC or its release from DnaB (Makowska-Grzyska & Kaguni, 2010). Secondly, helicase loading involves two regions of DnaA located in Domains III and Domain I. The region in Domain III has been identified based on studies of a monoclonal antibody that interferes with the interaction between DnaA and DnaB measured in solid-phase binding assays (Marszalek & Kaguni, 1994; Sutton et al., 1998). This antibody recognizes an epitope within residues 111-148 of DnaA. Other studies involving deletion analysis have also established the interaction region to

amino acids residues 135-148 which fall within the range of Domain III (Seitz, Weigel, & Messer, 2000). For Domain I, it has been shown that an alanine substitution for phenylalanine at position 46 affects the interaction between DnaA and the helicase DnaB (Keyamura et al., 2009). Thirdly, DnaC is unable to escort DnaB at unwound *oriC* by mutant DnaAs that are defective in self-oligomerization (Felczak & Kaguni, 2004; Felczak, Simmons, & Kaguni, 2005).

### 1.3.5. Dissociation of DnaB

DnaB must dissociate from DnaC in order to be available to unwind the DNA duplex. The dissociation of DnaC from the C-terminal domain of DnaB is enabled by the primase (DnaG) which binds to the N-terminal domain of DnaB (Bailey et al., 2007; Tougu & Marians, 1996; Wu, Zechner, & Marians, 1992). Most DNA polymerases require a primed ssDNA substrate to initiate DNA synthesis and priming is ensured by DnaG which synthesizes RNA primers by using the ssDNA as template (Corn & Berger, 2006). It has been observed that the interaction of primase with DnaB while it synthesizes a primer alters the conformation of DnaB which leads to the dissociation of DnaC (Makowska-Grzyska & Kaguni, 2010). The role of ATP in this dissociation reaction has been demonstrated (Makowska-Grzyska & Kaguni, 2010). It has been observed that substituting a conserved arginine for alanine in DnaC renders this enzyme defective in ATP hydrolysis and unable to dissociate from DnaB. It is thought that the conserved arginine acts to transduce the signal generated by the interaction of primase with DnaB and primer formation which leads to the release of DnaC from DnaB, via ATP hydrolysis by DnaC (Makowska-Grzyska & Kaguni, 2010).

### 1.3.6. Replication post-initiation

The replisome that duplicates the chromosome in *E. coli* is an ensemble of DnaB, the DNA polymerase III holoenzyme and primase. DNA polymerase III is a multi-enzyme complex with several subunits (Johnson & O'Donnell, 2005). Because of the anti-parallel structure of the duplex and the unidirectional activity of the DNA polymerases, DNA

replication is semi-discontinuous. One strand (leading) is synthesized continuously while the other strand (lagging) is synthesized discontinuously as Okazaki fragments.

Once DnaB is liberated from DnaC, it translocates in the 5'→3' direction on the lagging strand as it unwinds the parental duplex. To begin this process, two DnaB hexamers are introduced on the unwound region at the origin. Subsequent replication fork assembly requires the recruitment of the DNA polymerase III holoenzyme and its subsequent link-up with DnaB (Hiasa & Marians, 1994c). By transiently associating with DnaB, primase synthesizes a primer-template for the holoenzyme. The binding of the holoenzyme to the synthesized primer must happen fast so that there is enough time to establish the protein-protein interaction between the τ subunit of the holoenzyme and DnaB before the latter enzyme move away to perform her helicase functions. This interaction stimulates the rate of DNA unwinding catalyzed by DnaB by 15 fold and holds the holoenzyme to the fork (Kim et al., 1996., McHenry, 2003; Pomerantz & O'Donnell, 2007). It also designates which of the two DNA polymerase cores (each consisting of the α, ε, and θ subunits) of the holoenzyme becomes the leading-strand polymerase (Yuzhakov et al., 1996). On the lagging strand template, primase interacting with DnaB synthesizes primers for Okazaki fragment synthesis (Lu et al., 1996; Tougu & Marians, 1996; Wu et al., 1992). Primase remains attached to the RNA primed site through its interactions with SSB and is eventually displaced by the χ subunit (structural gene *holC*), one of the subunits of the clamp loader or γ complex. Following the displacement of primase, the γ complex then assembles the β clamp onto the primed site onto which DNA polymerase III core associates to form the processive polymerase III (Yuzhakov et al., 1999). The interactions of χ subunit with SSB tether Pol III to the lagging strand of replication and increase the processivity and efficiency of replication (Marceau et al., 2011; Witte et al., 2003). The SSB mediated switch between primase and polymerase frees primase to be recycled and be available to prime a new Okazaki fragment (Yuzhakov et al., 1999). Studies have shown that mutations in *holC* the structural gene for χ subunit leads to a defect in the initiation of DNA replication (Marceau et al., 2011). Other studies have also shown that a mutation in *holC* could decrease the dynamic exchange in polymerase subunits generating replication forks with decreased processivity and efficiency (Nordman, Skovgaard, & Wright, 2007).

### 1.3.7. Regulation of DNA replication initiation via origin sequestration

As stated above, regulation of DNA replication initiation is required in order to ensure that replication takes place only once every cell cycle and simultaneously from all the origins present in the cell (Katayama et al., 2010; Skarstad et al., 1986). *E. coli* achieves this feat by using *oriC* and DnaA as targets for regulatory control. In *E. coli*, studies have shown that after initiating each new round of DNA synthesis, the pre-replicative complex is disassembled in order to reset the origin (Nievera et al., 2006). In origin resetting, some of the displaced DnaA reassociates with the high affinity sites R1, R2 and R4 in the newly replicated copies of *oriC*, while the lower-affinity sites are blocked by the SeqA protein to prevent the formation of the pre-replicative complex (Nievera et al., 2006). The *oriC* region of *E. coli* contains GATC sequences that are found in DUE and in the lower-affinity sites R5M, τ1, τ2, I2, and I3. These sequences are normally methylated at the adenine nucleotide by Dam methyl-transferase, but immediately following their replication, these sites are transiently hemimethylated (Zyskind & Smith, 1986). SeqA binds the hemimethylated GATCs with high affinity (Slater et al., 1995) thus blocking pre-replicative complex reassembly without however impeding rebinding of DnaA at the high affinity sites R1, R2 and R4 (Nievera et al., 2006). Studies have shown that *oriC* is refractory to new initiations as well as adenosine remethylation of GATCs by Dam methylase for approximately one-third of the cell cycle, a time referred to as the sequestration period (Campbell & Kleckner, 1990). *seqA* deletion leads to the abolition of origin sequestration, which in turns leads to asynchronous replication, premature initiation and abnormal localization of nucleoids. All these problems emphasize the many functions of SeqA in regulating DNA replication (Boye et al., 1996; Lu et al., 1994; Olsson et al., 2003; Slater et al., 1995). In *seqA* mutants, overinitiation is due to the binding of the released DnaA-ATP to low affinity sites in *oriC* ensuring the reassembly of the pre-replicative complex (Nievera et al., 2006). Interestingly, in a wild type strain, SeqA also binds to the hemimethylated GATCs in the *dnaA* promoter region during the sequestration period, shutting down *dnaA* gene expression (Nievera et al., 2006).

### 1.3.8. Regulation of replication initiation via DnaA availability

The regulation of DnaA activity and availability is tied to replication fork movement and DNA sequence elements. New replication forks continue to move around the chromosome during the sequestration period when initiation and DnaA activity are blocked (Campbell & Kleckner, 1990). DnaA activity is regulated in three different ways as a result of fork movement. These regulatory routes are RIDA (regulatory inactivation of DnaA) a mechanism whereby DnaA-ATP bound to the chromosome is inactivated (Katayama & Sekimizu, 1999), duplication of sites on genomic DNA that have a high affinity for DnaA binding and thus titrates DnaA to reduce its availability (Kitagawa et al., 1998), and the duplication of specialized chromosomal regions known as DARS (DnaA recharging site) (Fujimitsu, Senriuchi, & Katayama, 2009). Even though the immediate assembly of the pre-replicative complex is prevented by sequestration, that is not enough to ensure that replication starts once and only once per cell cycle. Reducing the levels of DnaA-ATP coupled with sequestration is necessary. The conversion of DnaA-ATP to DnaA-ADP in *E. coli* is achieved by RIDA (Katayama et al., 2001) that is mediated by the Hda protein through its stable association with the sliding clamp of the DNA polymerase holoenzyme (Kato & Katayama, 2001). A conserved motif (arginine finger) in Hda interacts directly with the ATPase region of DnaA to stimulate ATP hydrolysis (Nievera et al., 2006). Studies have shown that ADP activates the *in vivo* activity of Hda (Su'etsugu et al., 2008). The ADP bound to Hda promotes the activated conformation of the protein which is a monomer. This monomeric form enables the arginine finger of the Hda $AAA^+$ domain to access the ATP bound to the DnaA $AAA^+$ domain (Su'etsugu et al., 2008). Lack of Hda activity produces a variety of phenotypes ranging from excessive overinitiation (Camara et al., 2005; Kato & Katayama, 2001) to modest overinitiation and asynchrony (Riber et al., 2006). However, less severe phenotypes have been attributed to compensatory mutations in Hda defective strains (Riber et al., 2006). It has also been observed that in some Hda mutants, the level of DnaA-ATP is almost equal to that seen in wild type cells (Fujimitsu et al., 2009) suggesting that other yet unidentified mechanisms that stimulate DnaA-ATP hydrolysis exist. Another mechanism utilized by the cell to control initiation is

to titrate free DnaA in order to reduce its availability. In fact, in *E. coli*, there are more than 300 consensus 9-mer DnaA recognition sites spread around the genome (Roth & Messer, 1998) that perform this function. One of such recognition site is the *datA* (DnaA titration) locus that titrates exceptionally large amounts of DnaA (Kitagawa et al., 1998). *datA* is believed to be the major DnaA titration locus on the chromosome. *datA* is located at 94.7 min on the genetic map (Kitagawa et al., 1996) and is duplicated near the end of the sequestration period. In fact, sequestration and the titration of DnaA by *datA* are two separate mechanisms and each operates independently with the latter working after the former to assure a single initiation event in the cell cycle (Kitagawa et al., 1998). Inactivating *datA* causes early initiation (Nozaki, Yamada, & Ogawa, 2009) and extra copies of *datA* are not tolerated by *E. coli* (Morigen et al., 2001).

Even though the cell has put in place mechanisms to regulate DnaA availability, when it is time to initiate replication, DnaA must be made available at *oriC*. Though DnaA-ATP is produced *de novo*, additional DnaA-ATP is also supplied by mechanisms that recharge DnaA-ADP. It has recently been discovered that specific genomic sequences termed DARS (Fujimitsu et al., 2009) located on distinct chromosomal regions stimulate the production of DnaA-ATP from DnaA-ADP. These sequences DARS1 and DARS2 have been shown to map near *bioD* and *mutH* respectively (Fujimitsu et al., 2009). These chromosomal regions have DnaA recognition sites that promote the regeneration of DnaA-ATP from DnaA-ADP, by nucleotide exchange. Any alteration to the DARS region such as mutations or deletions affects the cell cycle by delaying initiation (Fujimitsu et al., 2009). Recharging DnaA-ADP is not only limited to DARS sites. Other mechanisms of recharging are also available and one such mechanism involves the interaction of DnaA-ADP with membrane acidic phospholipids (Garner & Crooke, 1996). Interestingly it has been found that both DnaA and hemimethylated *oriC* sequences interact with membranes (Boeneman et al., 2009; Ogden, Pratt, & Schaechter, 1988; Saxena et al., 2013). These interactions may facilitate DnaA recharging at some point during the cell cycle.

## 1.4. Role of DNA topoisomerases in DNA replication

DNA topoisomerases are involved in the various stages of DNA replication from initiation to termination. They ensure the smooth sailing of the replication forks by removing DNA entanglements that will otherwise stall the progression of the replication forks. Their involvement in the various stages of replication ensures the smooth replication of DNA.

### 1.4.1. Role of DNA topoisomerases and supercoiling in replication initiation

In addition to the requirement of DnaA (Fuller et al., 1981), initiation of DNA replication in *E. coli* also requires supercoiled *oriC* DNA and it is in this regard that changes in the level of DNA supercoiling or transcriptional activity near *oriC* affects origin function and initiation timing (Baker & Kornberg, 1988; Skarstad et al., 1990). Using an *in vitro* replication system, it has been shown that the timely release of both replication forks for bidirectional replication requires topological modulation of the origin and this is achieved in the presence of either gyrase or topo IV (Smelkova & Marians, 2001). In *E. coli* the specificity of replication initiation for DnaA and the *oriC* sequence is maintained by topo I (Kaguni & Kornberg, 1984) and RNase HI (Ogawa et al., 1984).

Transcriptional activation of the origin has also been demonstrated in the phage λ. Initiation of phage λ replication requires transcription near its replication origin and this transcriptional activation depends on the transcription process itself rather than on any resulting protein product. Interestingly, transcription can start 95 bp downstream from *oriλ (*Furth et al.,1982). Negative supercoiling in light of the twin-supercoiled model of transcription (Liu & Wang, 1987) may play a role in activating the phage λ replication origin. That transcription is a major player in regulating *oriC* activity via negative supercoiling is confirmed by the direct interaction between DnaA and RNA polymerase (Flatten et al., 2009) and also between the replication initiation protein of *λ* phage, the *λ*O protein, and RNA polymerase (Szambowska et al., 2011).

Because replication initiation depends on local supercoiling, it is therefore regulated by DNA topoisomerases. In fact, physiological studies support the view that DNA topoisomerases and supercoiling are involved in replication initiation in *E. coli*. Deleting *topA* suppresses the temperature-sensitivity of *dnaA46* mutation (Louarn et al., 1984). Loss of topo I activity increases negative supercoiling (Pruss et al., 1982) which again increases the likelihood of stable strand separation. The addition of high concentrations of sodium chloride in a culture medium has also been shown to suppress the growth defect of *dnaA46* mutants by increasing negative supercoiling (Kondo et al., 2000). The inability of a gyrase mutant to initiate replication at non-permissive temperatures can be corrected by a *topA* deletion which increases negative supercoiling (Usongo et al., 2013). Studies using temperature sensitive *dnaA* initiation mutants have also provided evidence for the involvement of gyrase in replication initiation. A *dnaA46* mutant growing at the permissive temperature is three to four times more sensitive to gyrase inhibitors than a wild-type strain (Filutowicz, 1980). In addition, spontaneous novobiocin or nalidixic acid resistant mutations arise in *dnaA46* strain at a frequency 20 to 30 times lower than observed in the wild type strain (Filutowicz, 1980). More importantly, a *dnaA46* mutation is suppressed by an RNA polymerase mutation (*rpoB*). This double mutant exhibits three to fourfold lower sensitivity to novobiocin than the strain with only the *dnaA46* mutation alone (Filutowicz & Jonczyk, 1981). Studies have also shown that overinitiation in a *dnaAcos* mutant can be corrected by inhibiting DNA gyrase (Johnsen et al., 2010).

### 1.4.2. Role of DNA topoisomerases in the early stage of replication fork elongation

For DNA replication to proceed beyond initiation, the topological linkages ahead of the replication fork must be removed. Removal of each duplex turn as the parental template is unwound generates a compensatory positive overwinding ahead of the fork that can be manifested in two forms: a positive supercoil that forms in the unreplicated region ahead of the replication fork or a precatenane, a positive winding of the two partially replicated sister strands behind the fork. Accumulation of the positive supercoils ahead of

the fork will render further separation of the strands a daunting task and this will eventually stall the progression of the replication fork (Vos et al., 2011).

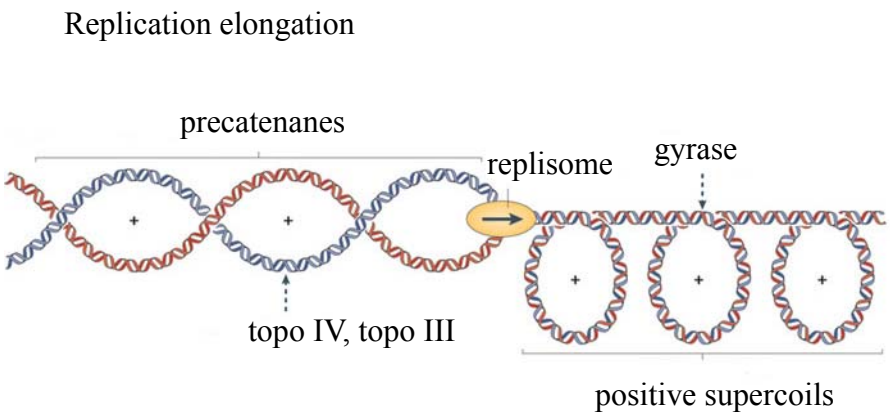


**Figure 11. The functions of DNA topoisomerases during replication elongation.** The progression of the replisome creates positive supercoils ahead of the fork and behind the fork where newly replicated strands form precatenanes. Positive supercoils and precatenanes must be taken care of in order to enable fork progression and proper chromosome segregation. Figure adapted with permission from Nature Publishing Group (Vos et al., 2011).

In *E. coli*, gyrase shoulder the responsibility to remove positive supercoils and topo IV supports gyrase in this role. The best evidence for this division of labor comes from a study assessing replication fork progression in temperature sensitive mutants of *S. typhimurium* (Khodursky et al., 2000). In this study, topo IV supports replication fork progression at one-third of the rate observed when gyrase and topo IV are present. Thermal inactivation of the gene encoding the A subunit of topo IV had very little effect on fork movement. By contrast, thermal inactivation of the gene encoding the A subunit of gyrase alone leads to a slow cessation of the replication fork. Thermal inactivation of the genes encoding the A subunits of both enzymes leads to rapid cessation of the replication fork. Several other lines of experimental evidence have equally pointed to gyrase and topo IV as the key topoisomerases in replication fork elongation *in vivo*. It has been found that the *parE10* (Ts) mutation which renders *E. coli* thermo sensitive for growth is lethal at all temperatures when PriA, the main replication restart protein, is absent (Grompone et al., 2004) implying that topo IV acts prior to replication completion even in the presence of

gyrase. Mutational inactivation of gyrase by shifting thermosensitive mutants of gyrase to the nonpermissive temperature causes DNA replication to stop only slowly. If gyrase was solely responsible for replication fork movement, the stop would be rapid (Filutowicz, 1980; Mirkin & Shmerling Zh, 1982; Orr et al., 1979). Other evidence has come from experiments with antibacterial drugs which have been shown to target both gyrase and topo IV and convert both enzymes into a poisonous road block on the DNA template that stall replication fork progression (Drlica et al., 2008, 2009).

Another consequence of replication fork progression is the formation of precatenanes as stated above. Evidence for the existence of precatenanes as an important substrate for topoisomerase action came from experiments whereby topo III was found to support the complete replication and segregation of *oriC* plasmids in an *in vitro* system consisting entirely of purified proteins (Hiasa & Marians, 1994a). Because topo III is a type 1A topoisomerase (Hartman Chen et al., 2013) it cannot relax positive supercoils, implying that topo III acts behind the fork to remove precatenanes by having access through the nicks or gaps generated during Okazaki fragment synthesis (Nurse et al., 2003). The removal of these precatenanes during replication fork progression is the principal responsibility of topo IV (Zechiedrich & Cozzarelli, 1995). In fact, studies have shown that precatenanes are responsible, at least in part, for *E. coli* sister chromosome cohesion, and impairing topo IV activity leads to increase cohesion time and failure to segregate newly replicated loci (Wang et al., 2008).

The role of topo IV in replication fork progression has also been questioned in some studies. In a study using a temperature sensitive mutant of topo IV, it has been observed that inactivating topo IV has a minimal effect on replication fork progression (Wang et al., 2008). Using an *in vitro* system consisting of entirely purified proteins to reconstitute *oriC* plasmid DNA replication as a model system to study the action of DNA topoisomerases, it was found that gyrase supported the early stages of replication whereas topo IV was important only at the terminal stages of replication (Hiasa & Marians, 1996). More importantly, it has been observed that decreasing gyrase activity slightly with a temperature sensitive *gyrB* mutant held at the semi-permissive temperature causes cell growth to be completely dependent on the replication restart protein PriA (Grompone et al., 2003). In

this study, the authors suggested that the partially inactivated gyrase was unable to keep pace with the generation of positive supercoiling which eventually stalls the replication fork. This suggests that topo IV does not compensate for the near inactive gyrase during replication fork progression to remove positive supercoils or precatenanes and also suggests that precatenanes are no barriers to replication. Interestingly, it has been reported that the activity of topo IV is temporally and partially regulated in the cell implying that topo IV is not free to bind DNA at all the times and can act only at a certain time in the cell cycle (Espeli et al., 2003). In fact, this temporal regulation has been confirmed experimentally using an isogenic pair of strains in which the A subunit of gyrase was either resistant or sensitive to norfloxacin. Norfloxacin targets both gyrase and topo IV (Khodursky et al., 1995). Cell killing by norfloxacin with the gyrase sensitive allele occurs with the onset of replication while cell killing when gyrase is resistant to norfloxacin occurs only late in the cell cycle implying that topo IV was restricted from gaining access to the DNA until only at the end of the cell cycle (Espeli et al., 2003). Irrespective of all these studies suggesting where topo IV or gyrase could possibly act in *E. coli*, the major function of topo IV in the cell is the removal of precatenanes and the decatenation of daughter chromosomes at the end of replication.

### 1.4.3. Role of DNA topoisomerases in the late stages of replication fork elongation

Positive supercoiling generated ahead of the replication fork becomes problematic near the end of the elongation step when the two replication forks converge for not only is the level of positive supercoil accumulation very high, the space between the two forks representing the stretch of unreplicated DNA is not long enough for the binding of gyrase (Wang, 2002). Depending on the speed at which the helicase unwinds the parental DNA, two pathways have been proposed to help replicate this region between the two converging replication forks (Wang, 1991). In pathway A, replication is completed before the unravelling of the intertwined parental strands. As result, the remaining intertwined parental strands are converted to intertwine between the newly replicated chromosomes forming catenanes. Type II DNA topoisomerases are needed to unlink the intertwined

duplex molecules that are formed in pathway A. In fact in *E. coli*, the Par- or partition defect phenotype (formation of large unsegregated nucleoid in midcell) has been observed in gyrase and topo IV mutants of *E. coli* suggesting that the action of a type II DNA topoisomerase is required in this pathway (Adams et al., 1992; Usongo et al., 2013; Wang et al., 2008). In pathway B, unraveling of the parental DNA occurs before replication. In this pathway, a type IA DNA topoisomerase can bind to ssDNA region and unlink the parental strands forming a pair of gapped but unlinked progeny DNA (Wang, 1991). However, due to the fact that single strands DNA regions form only transiently *in vivo*, it is very difficult to pinpoint the exact site of action of a type IA *in vivo*. As previously mentioned, the partnership between a type IA topoisomerase and a RecQ helicase in resolving converging replication forks has been demonstrated *in vitro* (Suski & Marians, 2008). This partnership may explain how a type IA may function *in vivo*. In eukaryotes, this partnership is also present and the RecQ family partner of topo III which is BLM is essential (Chan et al., 2007). Also justifying the partnership between a type IA topoisomerase and RecQ helicase is the natural fusion of a helicase domain and a topoisomerase domain in reverse gyrase which can also resolve convergent replication forks (Confalonieri et al., 1993; Declais et al., 2000; Hsieh & Plank, 2006).

In *E. coli* it has been suggested that topo I and topo III may participate in pathway B (DiGate & Marians, 1988; Minden & Marians, 1986). The utilization of the pathway B can also be inferred from some *in vivo* observations. It has been shown that temperature sensitive alleles of topo IV encoding subunits can be rescued by overexpressing topo III (Nurse et al., 2003; Perez-Cheeks et al., 2012).

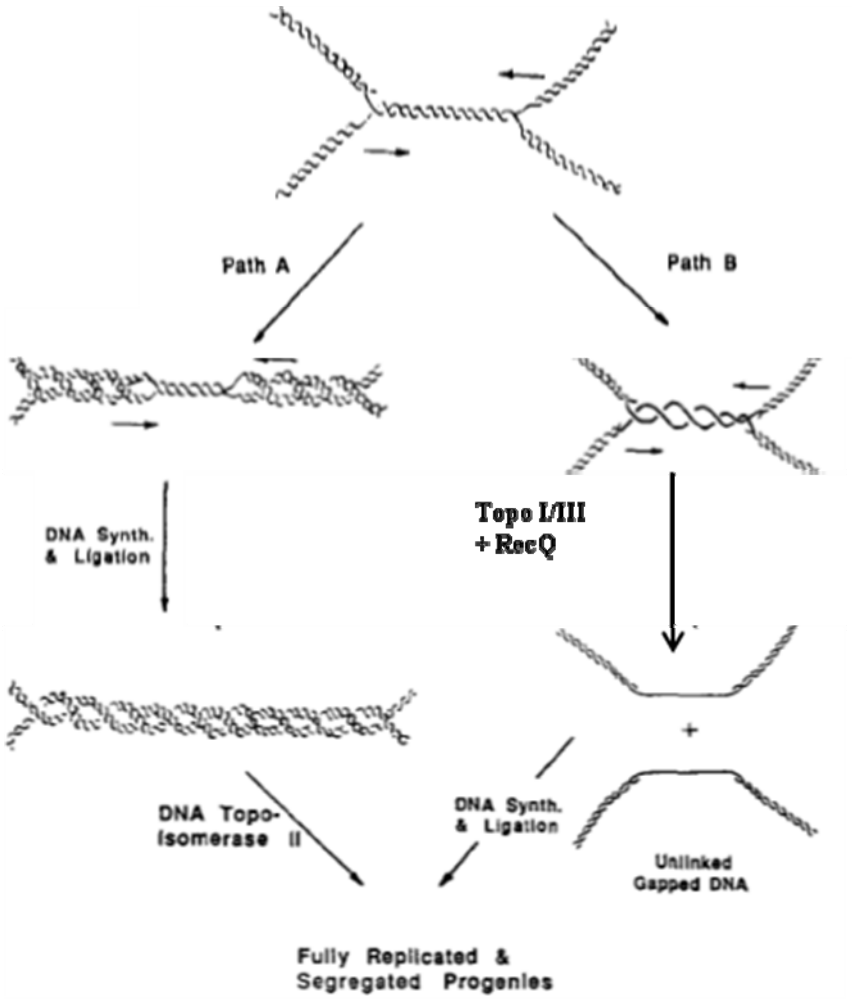


**Fig 12: Two pathways showing the merging of a pair of converging replication forks.** In pathway A, replication is completed before unravelling of the intertwined parental strands which are then converted to intertwine between the newly replicated chromosomes forming catenanes. A type II topoisomerase (topo IV) performs decatenation leading to fully segregated chromosomes. In pathway B, unlinking of the parental DNA strands precedes their replication. In this pathway, a type IA DNA topoisomerase can ensure unlinking of the parental DNA by binding to ssDNA regions. Type IA DNA topoisomerase could be recruited to the ssDNA region through their interactions with SSB and RecQ (Wang, 1991). Figure adapted with permission from the American Society for Biochemistry and Molecular Biology (ASBMB).

### 1.4.4. Topoisomerases and replication termination

The two replication forks that originate from *oriC* and traverse the chromosome bidirectionally meet at defined regions opposite of *oriC* called the termination region or *ter* sites. This region blocks the replication fork moving in one direction but not the other, by essentially creating a "replication fork trap" which is a one way ticket that allows the fork to enter but not to leave the terminus region (Duggin et al., 2008). In *E. coli*, the replication termination protein Tus recognizes and binds to the *ter* sites. In a situation whereby the progression of one fork is delayed, the other fork will encounter the *ter* site bound by Tus. In this situation, fork replication ceases until the second replication fork arrives (Neylon et al., 2005). The Tus-Ter complex arrest DNA replication by blocking the replicative helicase DnaB when it approaches the complex, eventually leading to its dissociation from the DNA polymerase complex (Duggin et al., 2008; Neylon et al., 2005). A role for topoisomerase I in replication termination in relation to supercoiling has been demonstrated in *E. coli*. In this bacterium, it has been shown that, in the presence of a mutated topo I, the ability of the Tus-Ter complex to block the progression of the replicative helicase DnaB is greatly diminished (Valjavec-Gratian et al., 2005). The authors of this study propose that an increase in negative supercoiling as a result of the topo I mutation enhances the unwinding ability of DnaB and, as a result, the duration of the Tus-DnaB interaction is reduced thereby leading to a decrease in the activity of Tus. In eukaryotes, the role of DNA topoisomerases in replication fork termination has also been studied. In *S. cerevisiae*, Top2 works in concert with the helicase Rrm3 to resolve the torsional stress that arises from converging replication forks and by doing so, they facilitate the progression of replication forks across chromosomal termination regions (*TERs*). The *TERs* in *S. cerevisiae* contain fork pausing elements and the association of Top2 and Rrm3 at *TERs* ensure replication fork fusion, and prevents abnormal genome rearrangements or DNA breaks that could arise as a result of fork convergence at these sites (Fachinetti et al., 2010). In *E. coli,* even though the Tus-Ter complex has have been heralded for terminating replication, it has been shown that Tus is not essential for survival (Hill, 1992) and it is conserved only in closely related bacteria (Neylon et al., 2005). Recent studies in *E. coli* have proposed the *dif* site as an

alternative termination site (Hendrickson & Lawrence, 2007). Elements consistent with non-Ter blocking mechanisms exist near *dif*. These include the FtsK translocase which moves in the same direction as the replication fork towards *dif* and opposes replication fork movement between *dif* and TerC or TerA. Another element consistent with termination at *dif* is XerC/D which can block fork progression by forming a stable complex with *dif* (Hendrickson & Lawrence, 2007).

### 1.4.5. Role of DNA topoisomerases and supercoiling in chromosome segregation

Chromosome segregation in *E. coli* is ensured by protein motors and cytoskeletal elements that help to compact the daughter chromosomes and help to move them to the appropriate cellular space prior to cell division. FtsK, a powerful protein motor, plays an important role in chromosome segregation (Bigot et al., 2007; Reyes-Lamothe, Nicolas, & Sherratt, 2012). It translocates DNA at a very fast rate and reads chromosome polarity with the aid of chromosomal sequences termed KOPS on to which it loads. These sequences are oriented to enable FtsK to act only at the *ter* region (Bigot et al., 2007; Reyes-Lamothe et al., 2012). FtsK also couples dimer resolution (mediated by the Xer recombination system) to cell division by bringing the *dif* site in *ter* to the septal area via translocation. In fact, FtsK is part of the divisome and is among the first proteins to localize at midcell to aid in the recruitment of other protein components of the divisome (Bigot et al., 2007; Reyes-Lamothe et al., 2012).

Another important protein complex that helps in chromosome segregation is MukBEF. This protein complex is encoded respectively by the genes *mukB*, *mukE* and *mukF*. The MukBEF protein complex helps to fold and compact DNA into a more condensed form with the aid of supercoiling (Holmes & Cozzarelli, 2000; Petrushenko et al., 2006). FtsK and MukBEF are important players in chromosome segregation in *E. coli*. *ftsK* mutants are defective in chromosome segregation and septation (Yu et al., 1998). *mukB* mutants are defective in chromosome segregation (Niki et al., 1991). Double *muk ftsK* mutants are inviable (Yu et al., 1998).

DNA topoisomerases play an important role in chromosome segregation by assisting in DNA compaction and disentanglement, and this is achieved by a cooperative work with proteins involved in chromosome segregation. Several lines of experimental evidence support this collaborative work. In *E. coli*, a physical association between topo IV and FtsK has been reported and the activity of topo IV is stimulated by FtsK. The raison d'être of this association could be a way for topo IV to counteract the supercoils created as a result of translocation by FtsK and also to help unlink tangled regions of DNA (Bigot & Marians, 2010; Espeli et al., 2003). Topo IV has also been found to physically interact with the actin-like protein MreB and this interaction stimulates decatenation. *mreB* mutant strains are defective in chromosome decatenation, thereby suggesting that the interaction of topo IV and MreB is an additional force that equally promotes chromosome segregation (Madabhushi & Marians, 2009).

It has been found that the segregation defect observed in *muk* mutants can be corrected by mutations in the *topA* gene that increase the overall degree of negative supercoiling, thus suggesting that supercoiling facilitates chromosome segregation (Sawitzke & Austin, 2000). In addition, it has also been found that *muk* mutants are hypersensitive to the gyrase inhibitor novobocin (Onogi et al., 2000) and the correction of the chromosome segregation defect of *muk* mutants by *topA* can be reversed by reducing the cellular activity of gyrase with coumermycin, an analog of novobiocin (Onogi et al., 2000). These findings strongly suggest a link between DNA supercoiling and chromosome segregation and thus between the topoisomerases that regulate supercoiling and chromosome segregation. In fact, the relationship between chromosome segregation and supercoiling can be explained by various mechanisms. Firstly, experimental studies coupled with simulation analysis have revealed that supercoiling of a pair of linked DNA rings facilitate their segregation because supercoiling increases the probability of decatenation of the rings and decreases the probability of their catenation (Rybenkov et al., 1997). Secondly, in the case of the MukBEF complex, the finding that negative supercoiling favors the folding of DNA into a shape normally assumed by DNA in this complex may explain why this complex would be partially dispensable in *topA* mutants (Petrushenko et al, 2006). In summary, the role of negative supercoiling on chromosome segregation may

be indirect and the common denominator between the two processes may be DNA compaction.

## 1.5. Recombination dependent replication

The maiden journey undertaken by replication forks from the origin to the terminus in an attempt to replicate the *E. coli* chromosome is not always a smooth one. In fact, accumulated evidence now shows that replication forks do encounter obstacles such as nicks on the DNA template that may trigger them to collapse (Kuzminov, 1995; Michel et al., 2007). Replication fork barriers are not only limited to nicks as other obstacles such as non-B DNA structures including R-loops, protein-DNA complexes, or even collision between the replication and transcription machineries can also slow down or arrest replication forks (Hyrien, 2000). Some of these obstacles such as protein-DNA complexes or collusion between replication and transcription are cleared by replicative helicases such as Rep, DinG and UvrD acting ahead of the replisome to facilitate fork movement. In fact, in *E. coli*, they have been demonstrated to act at forks blocked by replication-transcription collisions (Boubakri et al., 2010). Rep is the most important of these three helicases since it is the only one required for normal replication. In *rep* mutants chromosome replication is twice slower than in wild-type cells (Lane et al., 1975). Rep interacts physically and functionally with the replicative helicase DnaB (Guy et al., 2009).

In *E. coli*, recombination proteins have also been shown to facilitate replication progression under various conditions of replication impediment (Michel et al, 2007). Recombination plays a critical role (Kreuzer, 2005; McGlynn & Lloyd, 2002b) in the processing of impeded replication forks prior to replication restart. Recombination dependent DNA replication has been suggested as a means of reinitiating DNA replication when the replication fork is disrupted for whatever reason (Asai et al., 1994). Studies using *E. coli* have revealed that in several replication mutants (for example *rep*, *dnaBts*), recombination proteins play a specific role by participating in a reaction termed replication fork reversal (RFR) (Michel et al., 2004). Positive supercoiling has also been shown to promote RFR (Postow et al., 2001). RFR is a mechanism whereby the newly synthesized strands are unwound from the daughter duplexes and base pair to form a Holliday junction

adjacent to a double-stranded (dsDNA) end. The dsDNA ends are generated following the annealing of the leading and lagging strand ends. In a cell, double stranded DNA ends are recognized by the RecBCD complex (figure 13B). This complex unwinds and degrades double-strand ended DNA simultaneously until it encounters the Chi (crossover hotspot instigator) site. Upon encountering this site, the activity of RecBCD is modified. Its 3'→5' nuclease activity is attenuated, whereas a weaker 5'→3' activity is activated and its helicase activity remains unaltered (Dillingham & Kowalczykowski, 2008). All these biochemical activities culminate in the loading of the RecA protein on the 3' ended ssDNA with Chi on the 3' terminus (Dillingham & Kowalczykowski, 2008). RecA plays a key role in the homologous recombination reaction that ensues. It binds to single-stranded DNA (ssDNA) forming a RecA-ssDNA filament. This filament then invades a homologous double-stranded DNA (dsDNA) and pairs with its complimentary strand to initiate the strand exchange reaction (Cox, 2003). This exchange is extended by RecA-promoted branch-migration (Cox, 2003) and results in the formation of a four-arm double-stranded DNA structure called a Holliday junction (HJ). In *E. coli*, this junction is recognized by two branched DNA specific helicases, RuvAB (Seigneur et al., 1998) and RecG (McGlynn & Lloyd, 2000). The RuvAB helicase (West, 1997) acts in concert with the Holliday junction specific endonuclease RuvC (Zerbib et al., 1998) to cleave the Holliday junction and complete the recombination process (West, 1997). The RecBCD-RecA-catalyzed strand-invasion forms a D-loop (a recombination intermediate formed when a single strand DNA invades a complementary duplex) adjacent to the HJ. Replication can then restart from this D-loop structure with the help of the replication restart protein PriA and its partners which act upon these structures (Kowalczykowski, 2000). The assembly of the replisome at D-loops by PriA essentially couples double strand break (DSB) repair with replication re-initiation (Kogoma et al, 1996). It has been proposed that DNA lesions that do not generate double strand break requires regression of the nascent DNA or migration of the replication machinery away from the lesion to allow repair or bypass of the lesion to allow replication restart (Michel et al, 2004). This type of damage utilizes the RecFOR repair system (figure 13A) which loads RecA on the ssDNA and the subsequent formation of the Holliday junction (Michel et al, 2007). The SSB (single-stranded binding protein) prevents the

binding of RecA to ssDNA *in vivo*. The RecF, RecO and RecR proteins catalyze the initial stages of recombinational gap repair by promoting the binding of RecA to SSB-covered single-strand gaps (Morimatsu & Kowalczykowski, 2003). The 5'-3' single-strand exonuclease RecJ acts in several cases prior to RecFOR to enlarge gaps and it is associated sometimes with the 3'-5' RecQ helicase (Han et al., 2006).

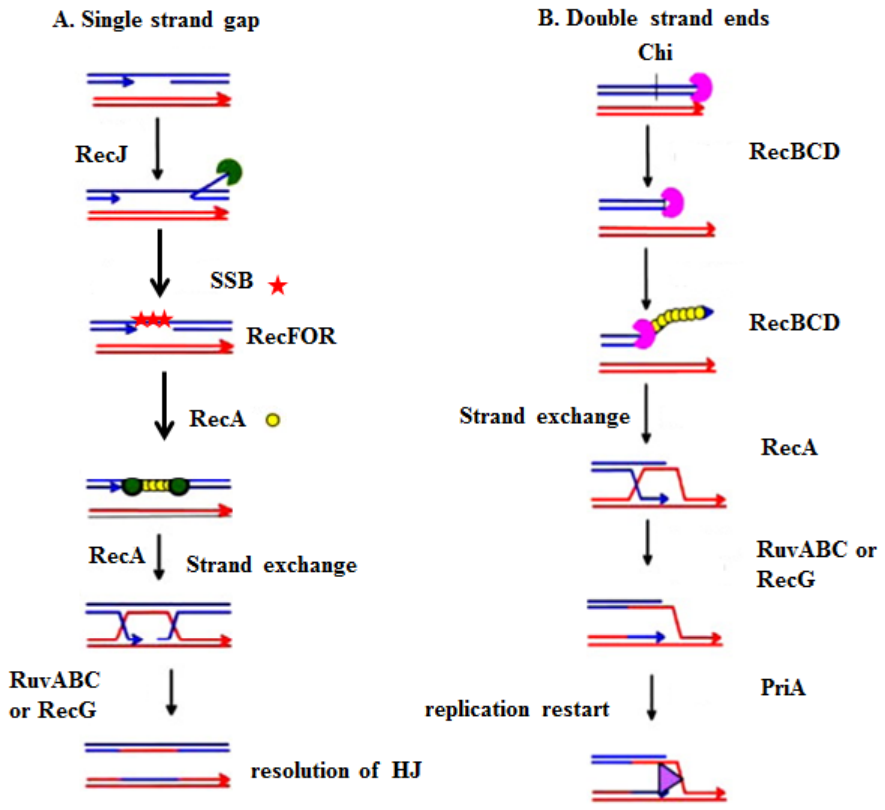


**Figure 13. DNA repair by homologous recombination and subsequent replication restart. A.** DNA gap repair by the RecFOR pathway. The 5'- 3' exonuclease RecJ enlarges the gap. SSB binds to single-strand DNA and prevents RecA loading. RecFOR promotes RecA loading on SSB-covered single-strand gaps. Strand exchange is promoted by RecA and the Holliday junctions generated are resolved by RuvABC or RecG.

**B.** Repair of double stranded ends by RecBCD which binds and degrades both strands until it reaches Chi. Upon reaching Chi, it modifies its activity and loads RecA on the 3'-ended single strand. RecA promotes strand-exchange and RuvABC or RecG terminates the recombination reaction by resolving the Holliday junction. D-loops generated are acted upon by PriA to load the replisome and restart replication. Blue and red lines represent DNA strands of two homologous molecules, arrow represents 3' ends. Green indented circle RecJ, green circles RecFOR, magenta indented circle RecBCD, yellow circle RecA, purple triangle PriA, red star SSB protein (Michel et al, 2007). Figure adapted and used with permission from Elsevier.

Holliday junction cleavage by RuvC can generate crossover and non-crossover products (Cromie & Leach, 2000; West, 1997). In a situation whereby crossover products are generated, subsequent restart and completion of replication will lead to the formation of chromosome dimers and this will necessitate the action of the *dif/xerCD/ftsk* system to resolve the dimeric chromosomes to monomers prior to cell division (Lesterlin, Barre, & Cornet, 2004). The generation of crossover products generates the risk of undesirable genetic exchanges so Holliday junction resolution that gives rise exclusively to non-cross over products is the best scenario for the cell. There is now a growing body of genetic evidence which indicate that this can be achieved with the action of a type IA topoisomerase working in conjunction with a RecQ-like family helicase (for example BLM in humans) to resolve homologous recombination intermediates such as double Holliday junctions (dHJs) without genetic exchanges (Ira et al., 2003).

Because type IA topoisomerases require single- stranded DNA regions for strand passage, (Hartman Chen et al., 2013), this makes their involvement in the resolution of double Holliday junction mechanistically challenging. Positive supercoiling stress within the dHJ diminishes the chances of single-stranded DNA formation, and also the unique topological challenges that this structure poses to the cell (Wang, 2002). The heteroduplex intertwines within a Holliday a dHJ cannot be readily converted to intertwine between double-stranded DNA segments that can be resolved by type II DNA topoisomerases. Moreover even though, practically the dHJ can be resolved by nucleolytic cleavage of DNA strands followed by rejoining of the DNA strands, this will generate recombinant DNA products (Wang, 2002). To overcome these challenges, a model has been proposed whereby topo IIIα and BLM work together in the step by step resolution of dHJ (Hartman Chen et al., 2013; Plank & Hsieh, 2009). In this model termed "unravel and unlink" the single stranded DNA region which is the preferred substrate for type IA topoisomerase is provided by the binding and subsequent unwinding of a Holliday junction by BLM aided by the single-stranded DNA binding protein RPA (replication protein A), the SSB homolog of eukaryotes, which can stabilize the ssDNA regions and that has also been shown to stimulate BLM activity through their physical interactions (Brosh et al., 2000).

Biochemical validation of this pathway has been demonstrated *in vitro* with the finding that model dHJs can be resolved with a human (Wu & Hickson, 2003) or *Drosophila* (Plank, Wu, & Hsieh, 2006) topoIIIα/BLM association.

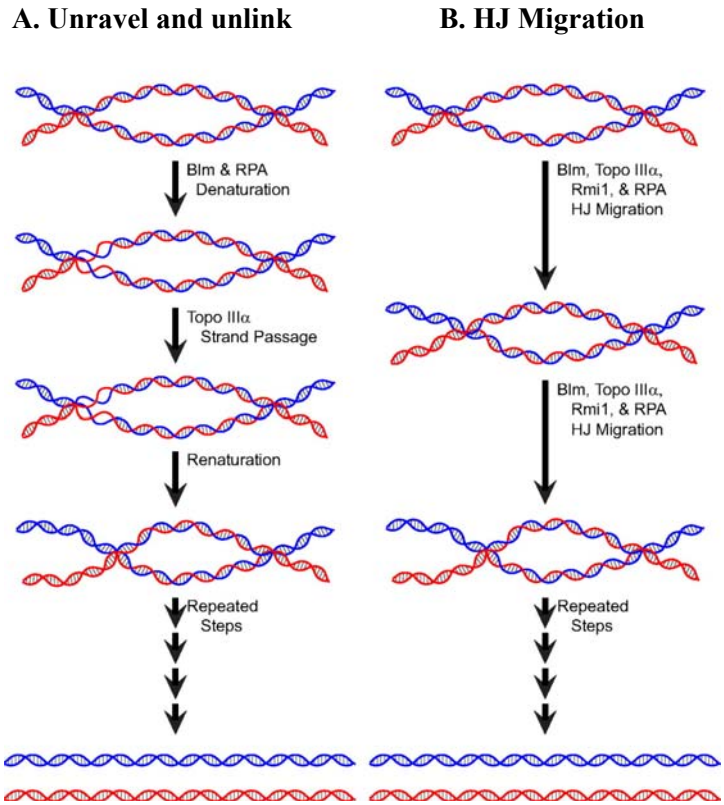


**Figure 14. Diagrammatic representation of two proposed models for the dissolution of dHJs. A.** In the first model unravel and unlink, BLM is recruited to a HJ with the help of single-stranded DNA-binding protein which is known as replication protein A (RPA) in eukaryotes. Binding of BLM to the HJ denatures a region of the heteroduplex in order to provide single-stranded DNA which is the preferred substrate for topo IIIα. Binding of topo IIIα enables strand passage. RPA dissociates and the denatured bubbles rewind with the exchange strand and the process is repeated until the HJ is resolved. **B.** In the second model termed HJ migration, dHJ dissolution is much more coordinated and processive. In this model, BLM enables the migration of the HJ while topo IIIα which is also bound to the complex comprising BLM, Rmi1 and RPA, perform coordinated strand passage (unlinking) on each heteroduplex. RPA helps stabilize the single-stranded DNA region necessary for topo IIIα catalysis while Rmi1 promote the activities of BLM and topo IIIα in the dissolution of the dHJ (Plank & Hsieh, 2009). Figure used with permission from the American Society for Biochemistry and Molecular Biology (ASBMB).

### 1.5.1. Replication restart

Stalled replication forks must be restarted and, because the stalling can happen anywhere on the chromosome for several reason as mentioned earlier, this implies that restart occurs through an *oriC*/*dnaA*-independent mechanism. Replication forks are restarted by replication restart proteins (Heller & Marians, 2006; Marians, 2000; Sandler & Marians, 2000) and these proteins include PriA, PriB, PriC, DnaT, DnaC, DnaB, and DnaG and they are collectively termed the primosomal proteins (Marians, 1992). The importance of replication fork restart to the cell is underscored by the existence of multiple genetic pathways to achieve this goal (Boonsombat et al., 2006; Sandler, 2000). PriA plays a key role in the assembly of the restart primosome (a protein complex that can assemble a replication fork independent of *oriC*/*dnaA*) (Marians, 2000). The importance of PriA in replication restart is validated by the severe phenotypes of *priA* mutants (Marians, 2000). These mutants grow poorly in rich media, filament extensively, have high basal levels of SOS expression, are UV sensitive and are also defective in homologous recombination, double strand break repair and in both inducible and constitutive stable DNA replication (Marians, 2000). The findings that *priA* mutant cells are viable though debilitated (Heller & Marians, 2005) may be explained in the context of the existence of multiple pathways to restart replication (Sandler, 2000). It has been found that cells that are deficient in PriA and PriC are not viable, thus supporting the implication of PriC in the PriA-independent pathway. Similarly, the finding that cells lacking both PriA and Rep are not viable indicates the importance of Rep in the PriA-independent pathway (Sandler, 2000). Several *dnaC* mutations have been identified as suppressors of the phenotypes of *priA* mutants and some replication restart mutants (Boonsombat et al., 2006; Sandler, 2000; Sandler, Samra, & Clark, 1996). *dnaC* can mutate at several positions to gain a function that allows the cell to restart replication in the presence of different combinations of *priA*, *priB*, *priC*, *dnaT* and *rep* mutations (Hinds & Sandler, 2004., Sandler et al., 1996., Sandler, 2005). One of such mutant *dnaC* alleles that suppress the phenotypes of PriA-null mutant (Sandler et al., 1996) encodes DnaC810. Biochemical characterization of DnaC810 reveal that it has gained the ability to load DnaB directly onto SSB-coated ssDNA, displacing SSB in the process and

bypassing the need for the PriA-directed primosome assembly that also requires PriB, PriC, and DnaT (Xu & Marians, 2000). *priB priC* double mutants are as defective as PriA-null mutants in homologous recombination and DNA repair following UV-damage. The phenotypes of double *priB priC* mutants can be partially corrected by the presence of the *dnaC809* mutation which encodes the same amino acid change as *dnaC810*, but requires a second *dnaC* suppressor mutation, two codons downstream of the first mutation, to regain wild-type activity (Sandler et al., 1996).

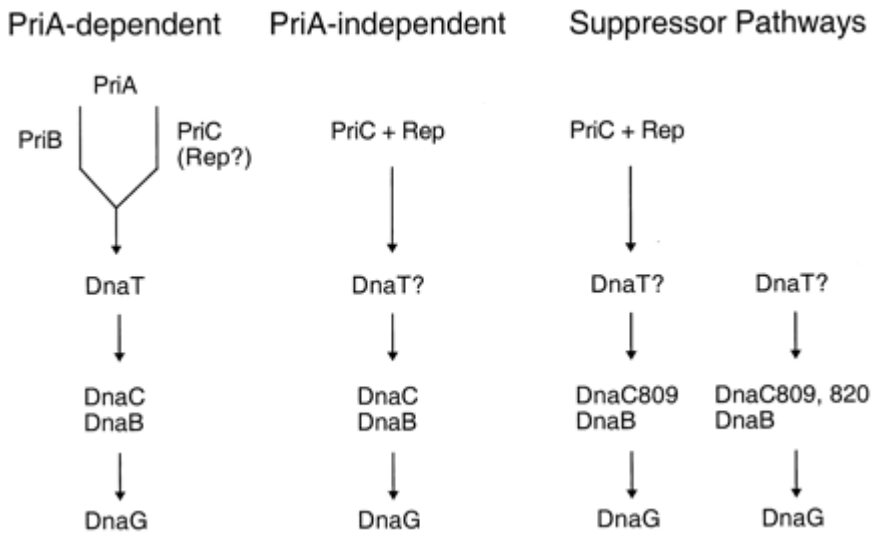


**Figure 15. Multiple genetic pathways to restart replication.** This figure shows the multiple genetic pathways that can be utilized to assemble a replication fork at a recombination intermediate. Each column represents one possible pathway. The steps separated by vertical arrows represent the order of action of the indicated proteins during the assembly of the primosome. Question marks indicate that the action of the protein in the pathway is unclear (Marians, 2000). Figure used with permission from the American Society For Microbiology (ASM).

Studies have shown that the choice of the restart pathway to be used is determined by the structure of the stalled replication fork, with the PriA-dependent system acting on fork structures with no gaps in the leading strand like the junction of a D-loop, while the PriC-dependent system uses fork structures with large gaps in the leading strand (Heller & Marians, 2005). In fact, it has been demonstrated *in vitro* that PriA can direct the assembly of a primosome on a D-loop DNA (Liu & Marians, 1999) and this ability of PriA

essentially links DNA replication and recombination (Jones & Nakai, 1999). To enable the loading of the primosome, the 3' end of the invading strand within the D-loop structure is bound by PriA. The loading of the replicative helicase DnaB and the primase DnaG onto the lagging strand template is then triggered by PriA through a series of protein-protein interactions (Sandler & Marians, 2000). The key step in fork reassembly is the loading of the primosome as this will enable the resumption of the unwinding of the parental strands via DnaB, thus enabling the synthesis of the RNA primers required for Okazaki fragments synthesis by DnaG. In addition, the 3' end can be used to prime renewed synthesis of the leading strand (Liu et al., 1999).

In support of the observation that the PriC restart pathway acts on gapped forks is the finding that *priA* mutants in which only the PriC restart system is functional requires the RecFOR gap-filling recombination proteins for viability (Grompone et al., 2004). The PriA restart pathway is also responsible to directly restart replication forks that disassembled by simple mechanical failure without necessitating repair by homologous recombination as observed in a gyrase thermo-sensitive mutant (Grompone et al., 2003). In this mutant, PriA is essential since double *gyrB priA* mutants are nonviable at the semi-permissive temperature and this lethality is not corrected by deleting *topA* implying that lethality is independent of the level of negative supercoiling. Inactivation of homologous recombination proteins has no effect on the viability of *gyrB priA* mutants indicating that the formation of recombination intermediates is not required for replication restart in double *gyrB priA* mutants. The lethality of double *gyrB priA* mutants can be suppressed by a *dnaC809* mutation which allows PriA-independent assembly of the primosome (Grompone et al., 2003).

## 1.6. Constitutive stable DNA replication (cSDR)

In *E. coli,* DNA replication generally initiates at *oriC.* In fact, *E. coli* possesses proteins that act as specificity factors to ensure that replication occurs only at *oriC*. These specificity factors are topo I (Kaguni & Kornberg, 1984) and RNase HI (Ogawa et al., 1984). Interestingly, both proteins have been shown to prevent R-loop formation (Drolet, 2006). In stationary phase *E. coli* cells, R-loops have been implicated in genome instability

(Wimberly et al., 2013) and this clearly underscore the importance of protecting the *oriC* based system of replication.

In *E. coli*, mutants were isolated that showed stable and constitutive chromosome replication (cSDR; replication can continue for a long time after protein synthesis inhibition) and that allowed them to support the deletion of the *oriC*/DnaA system (Kogoma, 1978; Kogoma & von Meyenburg, 1983). These mutants were subsequently found to be located in the *rnhA* gene which codes for RNase HI (Ogawa et al., 1984). Because *oriC*/DnaA-independent growth was observed in these mutants, studies were pursued to determine whether cSDR occurs at specific sites on the chromosome. Five sites or origins collectively termed *oriKs* were found to be used by *rnhA* mutants to initiate replication (de Massy, Fayet, & Kogoma, 1984; de Massy et al., 1984).

A model for cSDR has been proposed (Kogoma, 1997) and in this model, initiation is thought to begin at an R-loop generated by transcription. This bodes well with the identification of RNase HI as a specificity factor for *oriC* whose role was predicted to be the elimination of RNA transcripts hybridize to the DNA template that could serve as primers for DNA replication (Ogawa et al., 1984). In addition to RecA and DNA polymerase I, initiation of replication by cSDR also requires the complete assembly of the primosome initiated by PriA and the rest of the replisome (Masai et al., 1994). This bodes well with a recent model which suggests that replication restart proteins that load the replisome on a D-loop interact with substrates associated with cSDR to load the replisome (Sandler, 2005).

cSDR is not only limited to *rnhA* mutants. It has been shown that *E. coli* cells lacking *recG* exhibit cSDR (Hong, Cadwell, & Kogoma, 1995). However, in this case the *oriC*/DnaA system could not be deleted. It has been shown that RecG can unwind D-loop and R-loop structures (Fukuoh et al., 1997; McGlynn et al., 1997; Vincent, Mahdi, & Lloyd, 1996) and it was suggested that the persistence of R-loops in both *rnhA* and *recG* mutants is responsible for cSDR (Hong et al., 1995). It has also been found that damage induced SDR (iSDR) is partially responsible for the increase in SDR observed in cells lacking RecG (Hong et al., 1995). iSDR is a form of SDR that depends on the activation of the SOS response (Kogoma, 1997) and cells lacking RecG have been shown to exhibit a

mild SOS constitutive phenotype (Asai & Kogoma, 1994; Lloyd & Buckman, 1991; O'Reilly & Kreuzer, 2004). In addition, induction of SOS following thymine starvation leads to iSDR levels that are significantly higher in cells lacking RecG compared to wild type cells (Asai & Kogoma, 1994). More recent studies have shed more light on the role of RecG in SDR. RecG plays an important role in the control of chromosome replication and segregation in bacteria (Rudolph et al., 2009) and this is attributable to the ability of RecG to limit SDR (Kogoma, 1997; Rudolph et al., 2009; Rudolph, Upton, & Lloyd, 2009).

Several lines of experimental evidence support the role of RecG in SDR. Firstly, in mock-irradiated *dnaA46* single mutants shifted to 42ºC the temperature at which the mutant DnaA protein is inactive, it was found that incorporation of [$^3$H]thymidine continued for some time before reducing severely, consistent with the fact that only the forks that were already initiated terminated replication while new rounds could not be initiated from *oriC* because of inactive DnaA proteins. UV irradiation was found to increase the level of [$^3$H] thymidine incorporation in mock-irradiated wild type strains (Rudolph et al., 2009; Rudolph, Upton, & Lloyd, 2007) and this increase was in line with the induction of SDR defined by its independence of DnaA (Kogoma, 1997). In mock *dnaA46 recG* cells shifted to 42ºC, the level of incorporation was found to be significantly higher than in mock-irradiated *dna46* cells consistent with the increased levels of SDR reported in *recG* mutants (Hong et al., 1995). The level of incorporation following UV irradiation in *dnaA46 recG* cells was almost as high as in an irradiated *recG* single mutant implying that replication is affected in the absence of RecG, both with and without UV irradiation (Rudolph et al., 2009). Secondly, the origin and terminus areas of the chromosome in *dnaA46* and *dna46 recG* strains were tagged with fluorescent proteins (Rudolph, Upton, Harris, et al., 2009; Rudolph, Upton, & Lloyd, 2009) and, following UV irradiation at the restrictive temperature, a moderate accumulation of origin as well as terminus foci in *dnaA46* cells was observed after several hours, consistent with the presence of iSDR in these cells (Kogoma, 1997). UV irradiation on *dnaA46* cells lacking *recG* further increased SDR. Indeed, there was a rapid and dramatic multiplication of the origin and terminus foci in these cells and they formed extensive and discrete clusters within the filaments (Rudolph et

al., 2009). Interestingly, this effect has also been observed in *recG* cells in which origin (*oriC*) firing is functional (Rudolph et al., 2009).

The perpetuation of SDR has been attributable to secondary replication forks that are initiated as a result of pathological events that arise from unscheduled collisions between opposing forks (Hiasa & Marians, 1994b; Krabbe et al., 1997; Markovitz, 2005). RecG has been proposed to limit this secondary pathological amplification and this explains the elevated SDR and the defects in chromosome segregation and cell division observed in *recG* mutants (Rudolph et al., 2009). That RecG can exploit DNA damage as an initial trigger for pathological replication is supported by the observation that SDR can continue for an extensive period of time in *recG* cells regardless of the UV dose and, more importantly, a substantial reduction in UV dose did not impact the amplification of origin and terminus foci (Rudolph, Upton, & Lloyd, 2009). It has been recently proposed that every replication fork collision event has the potential to threaten genome integrity when RecG is absent. The replication restart pathway is exploited to re-replicate the chromosome in the absence of RecG with the potential to cause genome instability (Rudolph et al., 2013). Interestingly, most of the observed phenotypes of *recG* are suppressed by mutations in PriA helicase. Several suppressors of *recG* mutant phenotypes identified so far have single amino acid substitution within or near the helicase motif of PriA (Al-Deib, Mahdi, & Lloyd, 1996) and these PriA helicase mutations have been shown to effectively suppress the damage sensitivity of *recG* mutants (Al-Deib et al., 1996; Gregg et al., 2002; Jaktaji & Lloyd, 2003). Amplification of origin and terminus was dramatically reduced in UV irradiated *dnaA46 recG priA300* cells at 42$^{o}$C (Rudolph et al., 2009). This finding is in line with a previous observation that strains expressing a helicase-defective PriA protein show reduced levels of constitutive as well as damaged induced SDR (Tanaka et al., 2003). The fact that cells lacking PriA helicase activity suppressed the *recG* mutant phenotypes clearly demonstrates that the pathological replication in *recG* cells is linked to SDR.

## 1.7. Topoisomerase inhibitors

Because of their essential functions, topoisomerases are the principal target of most antibacterial and chemotherapeutic agents. In bacteria, due to the central role of gyrase in

the cell, it is not surprising that it is the target of an important variety of antibacterial agents. Mechanistically, gyrase inhibitors are classified into two broad categories. The first category which includes the coumarins and cyclothialidines acts by inhibiting the ATP dependent reaction of gyrase by preventing ATP hydrolysis, since they compete with ATP for binding to the GyrB subunit. This class of antibiotics only inhibits the supercoiling reaction of gyrase. The relaxation activity of gyrase is unaffected by this category of antibacterial compounds (Lewis et al., 1996; Maxwell, 1999). The synthetic quinolones comprise the second category and they function as gyrase poisons by stabilizing the enzyme-DNA covalent intermediates (Lewis et al., 1996; Maxwell, 1999). Stabilized enzyme-DNA adducts hinder the progression of replication and transcription complexes (Drlica & Zhao, 1997; Pohlhaus & Kreuzer, 2005). The release of DNA ends in the enzyme-DNA ternary complexes also leads to widespread chromosomal fragmentation resulting in rapid quinolone-mediated cell death (M. Malik et al., 2006). There are also naturally occurring proteinaceous gyrase inhibitors that inhibit gyrase by trapping the enzyme-DNA covalent intermediates. Prominent among this class is CcdB encoded by the F plasmid (Couturier, Bahassi el, & Van Melderen, 1998; Kamphuis et al., 2007). The F plasmid or F factor is a ring shaped DNA parasite in *E. coli*. This plasmid encodes proteins that ensure equal partition of F DNA progenies following division. In this system, when an *E. coli* cell of F plasmid progeny inherits both copies of the duplicated F plasmid, the plasmid-free *E. coli* is killed to stop it from producing descendants that are plasmid free. This is achieved by a toxin-antitoxin system strategy utilized by the F plasmid (Couturier et al., 1998; Kamphuis et al., 2007). This plasmid encodes two proteins, CcdA and CcdB, with the former being the antitoxin and the latter the toxin. CcdB is more stable than the labile CcdA which must be constantly replenished in *E. coli* cells bearing the F plasmid. This constant replenishment ensures that the complex formed between CcdA and CcdB remains harmless to the host cell. When the decaying CcdA cannot be replenished due to the loss of the F plasmid, the long lasting CcdB toxin kills the cell. In the absence of CcdA, CcdB kills the cell by turning gyrase into a DNA damaging agent. Studies have shown that in the CcdB-gyrase complex, the DNA gate in the gyrase-DNA complex cannot be regulated and stays permanently unlocked (Couturier et al., 1998; Kamphuis et al., 2007).

By doing so, the CcdB toxin prevents the rejoining of gyrase-linked DNA ends in the CcdB-gyrase-DNA ternary complex. Other *E. coli* plasmids also produce toxins that employ a strategy similar to that utilized by the F plasmid. They produce a toxin called microcin B17 (MccB17) which equally turns gyrase into a lethal enzyme by trapping the covalent gyrase-DNA complex (Vizan et al., 1991).

New endogenous proteinaceous gyrase inhibitors have been discovered that act differently from the classical gyrase inhibitors. These are proteins that interact with gyrase to modulate its activity. Bacteria may have evolved to adopt this strategy in order to guard against external agents that may take advantage of the topoisomerization reaction of DNA gyrase. One prominent proteinaceous gyrase inhibitor is YacG. NMR studies revealed the presence of a unique zinc finger motif and an unstructured tail in YacG (Ramelot et al., 2002). This protein was shown to be a member of the interactome of DNA gyrase based on protein- protein interactions network (Butland et al., 2005). Recently a function has been assigned to this protein which explains why it was found in the interactome of gyrase. YacG inhibits gyrase activity by binding to the carboxy terminus domain of the B subunit of gyrase, which makes the enzyme unable to bind to DNA (Sengupta & Nagaraja, 2008). This action of YacG targets only gyrase, as other topoisomerases such as topo I and topo IV are immune to the action of YacG (Sengupta & Nagaraja, 2008). YacG inhibition of gyrase protects cells from the cytotoxic effects of gyrase inhibitors which wreak havoc by corrupting the topoisomerization reaction of gyrase (Sengupta & Nagaraja, 2008).

Another endogenous gyrase inhibitor is glutamate racemase or Mur I (Ashiuchi et al., 2002). In *E. coli*, the dual function of MurI, referred as moonlighting, allows this protein to function as a gyrase inhibitor and as a racemization protein catalyzing the racemization of glutamate, by converting L-glutamate to D-glutamate, an essential component of the peptidoglycan. Whereas the racemization activity can moonlight as a gyrase inhibitor in *E. coli*, in *Mycobacterium tuberculosis*, racemization and gyrase inhibition have been reported as two independent activities of the protein (Sengupta, Ghosh, & Nagaraja, 2008). Mur I inhibits gyrase activity by binding to its A subunit and this prevents gyrase from gaining access to the DNA substrate. The sequestration of gyrase

by Mur I inhibit all reactions catalyzed by gyrase (Sengupta, Shah, & Nagaraja, 2006). The activity of Mur I is enzyme specific, as topo I activity is unaffected. Because Mur I is not a typical potent gyrase inhibitor, it has been proposed to act as a modulator of gyrase activity (Sengupta et al., 2006). In addition to Gyr I discussed in the literature review, other inhibitors that inhibit gyrase by interfering with enzyme-DNA interactions include MfpA from *Mycobacterium sp* (Hegde et al., 2005; Montero et al., 2001).

## 1.8. Rationale, hypotheses and objectives

DNA topoisomerases are essential enzymes that regulate and maintain the topological state of chromosomal DNA by making transient breaks, passing a DNA segment through the break and subsequently resealing the breaks. These enzymes are crucial in managing DNA topology during all macromolecular transactions on DNA (Wang, 2002). Their activities ensure that processes such as DNA replication and subsequent chromosome segregation are successfully executed without which the stability of the genome will be seriously compromised. In fact during replication, the excess positive topological linkages that accumulate that might otherwise block the progression of replication forks are removed by topoisomerases (Wang, 2002). Through their homeostatic regulation of supercoiling, DNA topoisomerases prevent the formation of R-loops. Topo I, a type IA topo relieve negative supercoils which can facilitate reannealing between the nascent transcript and the template DNA leading to the formation of R-loops (Drolet, 2006). R-loops can block the progression of the replication forks and are generally hotspots for genome instability (Drolet, 2006).

Because of these essential functions and their evolutionary conservation across bacterial species, DNA topoisomerase inhibitors are among the most common and most effective antibacterial drugs. These enzymes are targets of the pharmaceutical industry. Several classes of topoisomerase inhibitors namely the quinolones, coumarins and cyclothialidines work by corrupting the topoisomerization reaction, with the end result being the block of DNA synthesis and bacterial cell death (Drlica et al., 2008; Malik et al., 2006).

Gyrase is the principal target of most of these antibacterial drugs. Inhibitors of gyrase can either prevent the binding of ATP to gyrase thereby preventing supercoiling or stabilize the enzyme-DNA covalent intermediates thereby acting as roadblocks for DNA tracking machineries (Lewis et al., 1996; Maxwell, 1999). Bacteria have chromosomally encoded gyrase inhibitors that protect the cells against compounds that target gyrase. Most of them act by limiting the topoisomerization reaction of gyrase and reduce the efficacy of antibacterial drugs that target gyrase. An example of a chromosomally encoded gyrase inhibitor is GyrI. It has been proposed to act by sequestering gyrase and thereby limiting its effective concentration (Chatterji & Nagaraja, 2002). Other chromosomally encoded gyrase inhibitors may also be proteins that sequester ATP thereby affecting the supercoiling reaction of gyrase. Because most antibacterial drugs target topoisomerases, bacterial resistance to these therapies is one of the biggest challenges of the medical world. Most antibiotics in use are just a refinement of the antibiotics that were earlier discovered. The lack of interest in pursuing new targets has rendered the antibiotic cupboard bare (Walsh & Wright, 2005).

Even though there has been an effort to develop new targets recently, the spread of antibiotic resistance has rendered these therapies ineffective not only within community settings (Critchley et al., 2007; Jacobs, 2003) but also in hospital settings (Pong & Bradley, 2004; Prystowsky et al., 2001) and this resistance extend both to gram positive (Howell-Jones et al., 2005; Jones, 2010) and gram negative organisms (Kollef, 2005; Poole, 2005). The spread of antibiotics resistance justifies the need for new antibiotics (Silver & Bostian, 1993). The identification of new chromosomally encoded gyrase inhibitors may serve as attractive targets for the development of new antibacterial drugs. Identifying these targets may also help to combat antibiotic resistance. Because these chromosomally encoded gyrase inhibitors generally decrease gyrase activity, they render bacterial cell killing by topoisomerase inhibitors inefficient leading to the spread of antibiotic resistance. Targeting chromosomally encoded gyrase inhibitors will therefore improve the efficacy of antibiotics. As mentioned earlier, most of the antibacterial drugs developed so far target only the type II DNA topoisomerases. The ubiquitous presence and the numerous cellular functions of the type IA DNA topoisomerases will also make them attractive therapeutic targets.

In the first part of the project, we wanted to understand the link between R-loop formation and hypernegative supercoiling. According to the twin supercoiled domain model of transcription (Liu & Wang, 1987), domains of positive and negative supercoils are formed in front and behind the RNA polymerase complex respectively during transcription. Gyrase removes positive supercoils and introduce negative supercoils while topoisomerase I (*topA*) relaxes the transcription-induced negative supercoils that will otherwise cause the formation of R-loops (DNA/RNA hybrids) in *topA* mutants leading to excess negative supercoiling. In the cell, RNase HI (*rnhA*) is an enzyme that can remove the RNA moiety of an R-loop. R-loops can prime DNA replication independent of *oriC* via the mechanism known as cSDR (Kogoma, 1997) described earlier. We constructed a triple *topA rnhA gyrB*(Ts) mutant to better understand the between R-loop formation and hypernegative supercoiling. This triple mutant can grow if it carries a plasmid allowing for the conditional expression of RNase HI or topoisomerase III. Surprisingly, initial supercoiling analysis of this triple mutant revealed that the DNA of these cells was extensively relaxed instead of being hypernegatively supercoiled following the depletion of RNase HI. This seems to be related to a cellular response leading either to excess relaxation or supercoiling inhibition. Also, initial observation of these cells under the microscope following DAPI staining revealed cells with long filaments packed with unevenly distributed DNA consistent with chromosome segregation defects. These initial observations led us to put forward the following hypotheses:

-Supercoiling inhibition in triple *topA rnhA gyrB*(Ts) mutants is not linked to increase DNA relaxation activity by topoisomerase IV but to inhibition of supercoiling by gyrase.

-GyrI, a well-characterized chromosomally encoded gyrase inhibitor is not implicated in supercoiling inhibition in triple *topA rnhA gyrB*(Ts) mutants.

-Supercoiling inhibition in *topA rnhA gyrB*(Ts) mutants is not linked to the SOS response which is chronically expressed in the absence of *rnhA*.

-Gyrase inhibitors accumulate in *topA rnhA gyrB*(Ts) cells.

-The chromosome is over-replicated in *topA rnhA gyrB*(Ts) mutants.

To verify these hypotheses, we had the following objectives:

-To understand the mechanism of supercoiling inhibition in *topA rnhA gyrB*(Ts) mutants.

- As long term objective, to purify, characterize and study the regulation of the gyrase inhibitor(s) synthesized in *topA rnhA gyrB*(Ts) mutants.

To address the first objective, we first of all ascertained that the relaxation observed in *topA rnhA gyrB*(Ts) mutants was unrelated to topoisomerase IV activity. We inhibited the activity of topoisomerase IV with norfloxacin (norfloxacin targets both topo IV and gyrase but we used a gyrase allele that was resistant to norfloxacin so only topo IV was targeted) and we found through *in vivo* supercoiling analysis that the DNA of *topA rnhA gyrB*(Ts) mutant was still extensively relaxed. We also introduced the *smbC*::kan allele (*smbC* encodes GyrI) in the triple *topA rnhA gyrB*(Ts) mutant and following supercoiling analysis, we found that the DNA was still extensively relaxed. These results are presented in chapter two. Secondly, we performed a genetic screen by using the Tn5 transposon mutagenesis system developed by Metcalf and co-workers (Larsen et al., 2002) to identify suppressors of *topA rnhA gyrB*(Ts) cells that restored supercoiling and corrected the growth and chromosome segregation defects. We identified suppressors that restored supercoiling and corrected the growth and segregation defects of triple *topA rnhA gyrB*(Ts) mutants. Most of these suppressors were found in genes implicated in DNA replication. Part of the suppressor screen is presented in chapter four and the rest is presented in the appendix. The second objective is still to be realized.

The second part of the project is related to the function of type 1A topos in *E. coli*. It was initially based on our observations that *topA topB* mutants displayed growth and chromosome segregation phenotypes similar to those of triple *topA rnhA gyrB*(Ts) mutants. Because of these initial observations, we hypothesized that:

-In *E. coli*, type IA topoisomerases play important roles in chromosome segregation and genome maintenance by regulating replication.

-Suppressors of triple *topA rnhA gyrB*(Ts) mutants that map to genes that encode proteins implicated in replication, should correct the growth and segregation defects of *topA topB* mutants.

To verify these hypotheses we had as objective:

-To investigate the role of type IA DNA topoisomerases in chromosome segregation and genome maintenance.

In the first part of this section, we wanted to assess the role of type IA topos in chromosome segregation and genome maintenance. To do this, we instigated whether type IA topos could correct the phenotypes of a *gyrB*T(s) mutant at the nonpermissive temperature. Gyrase as previously mentioned is encoded by (*gyrA* and *gyrB*). This protein plays a key role in all the stages of DNA replication. We found that the Par- phenotypes and chromosome segregation defects of a *gyrB*(Ts) mutant at the nonpermissive temperature were corrected by deleting *topA* only when *topB* was present. Overproducing topoisomerase IV failed to correct the segregation defects in the absence of both type IA enzymes. These results are presented in chapter three. We also used genetic approaches combined with suppressor screens, spot assays and microscopy to further investigate the roles of type 1A topoisomerases in genome maintenance. We found that, in the absence of type IA topoisomerases, cells formed very long filaments packed with diffuse and unsegregated DNA. Compensatory mechanisms that corrected the growth and segregation defects in the absence of *topA* included deleting *recQ*, *recA,* and overproducing topoisomerase III, while compensatory mechanisms that corrected growth and segregation defects in the absence of both enzymes included overproducing RNase HI and deleting *recA*. Moreover, several of the suppressor mutations isolated from our *topA rnhA gyrB*(Ts) strain that affected replication, namely *oriC15::aph*, *dnaT18::aph*, *holC2::aph* and *rne59::aph*, alleviated to different extent the phenotypes of cells lacking topo I and/or lacking both topo I and III. These results are presented in chapter four. Altogether, our results shed light on the roles of type 1A topoisomerases, supercoiling and R-loops on genomic stability may help to identify new targets for antibiotics, and to have a better understanding of how unscheduled DNA transactions could lead to various genetic diseases and cancer.

# Preface to Chapter 2

One major function of DNA topoisomerase I (*topA*) is to inhibit R-loop formation by relaxing transcription induced supercoiling. The accumulation of excess negative supercoiling in *topA* mutants is attributed to R-loop formation coupled to gyrase activity. Overproducing RNase HI (*rnhA*) removes R-loops and suppresses the accumulation of hypernegative supercoils in *topA* mutants. It was predicted that the inability to construct *topA rnhA gyrB*(Ts) mutants was because such mutants would die from toxic levels of hypernegative supercoiling. We report here the construction of a conditional *topA rnhA gyrB*(Ts) mutant following the conditional expression of RNase HI from a plasmid borne gene. We characterize this mutant using fluorescence microscopy to look at DNA distribution and plasmid supercoiling analysis/gyrase assays in crude cell extracts to study gyrase supercoiling activity. Our results reveal that depleting RNase HI in a *topA gyrB*(Ts) mutant leads to supercoiling inhibition and chromosome segregation defects. The DNA of *topA rnhA gyrB*(Ts) cells is extensively relaxed. Overproducing topoisomerase III corrects the chromosome segregation defects without restoring supercoiling. RNase HI plays a major role in the control of DNA topology.

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Author's contribution.

The project was designed by Dr. Marc Drolet. My contribution in this project was significant. In this project, I performed half of the experiments. I prepared all the figures presented in the article and wrote some sections of the materials and method. I contributed to ideas towards the writing of the manuscript and did proofread the final draft

Dr Marc Drolet wrote the paper.

# CHAPTER 2: Manuscript I

**Depletion of RNase HI activity in *Escherichia coli* lacking DNA topoisomerase I leads to defects in DNA supercoiling and segregation**

**Running title: RNase HI in DNA topology**

Valentine Usongo, Flora Nolent, Patrick Sanscartier, Sonia Broccoli, Imad Baaklini, Karl Drlica and Marc Drolet

**Keywords:** *topA*, topoisomerase I, gyrase, RNase HI, segregation.

# Abstract

Gyrase-mediated hypernegative supercoiling is one manifestation of R-loop formation, a phenomenon that is normally suppressed by topoisomerase I (*topA*) in *Escherichia coli*. Overproduction of RNase HI (*rnhA*), an enzyme that removes the RNA moiety of R-loops, prevents hypernegative supercoiling and allows growth of *topA* null mutants. We previously showed that *topA* and *rnhA* null mutations are incompatible. We now report that such mutants were viable when RNase HI or topoisomerase III was expressed from a plasmid-borne gene. Surprisingly, DNA of *topA* null mutants became relaxed rather than hypernegatively supercoiled following depletion of RNase HI activity. This result failed to correlate with the cellular concentration of gyrase or topoisomerase IV (the other relaxing enzyme in the cell) or with transcription-induced supercoiling. Rather, intracellular DNA relaxation in the absence of RNase HI was related to inhibition of gyrase activity both *in vivo* and in extracts. Cells lacking *topA* and *rnhA* also exhibited properties consistent with segregation defects. Overproduction of topoisomerase III, an enzyme that can carry out DNA decatenation, corrected the segregation defects without restoring supercoiling activity. Collectively these data reveal 1) the existence of a cellular response to loss of RNase HI that counters the supercoiling activity of gyrase and 2) supercoiling-independent segregation defects due to loss of RNase HI from *topA* null mutants. Thus RNase HI plays a more central role in DNA topology than previously thought.

## Introduction

Negative DNA supercoiling is a regulated feature of bacterial chromosomes (Menzel and Gellert, 1983; DiNardo *et al*., 1982; Pruss *et al*., 1982; Drlica, 1992) that is important for most processes involving DNA strand separation. In *E. coli*, supercoiling regulation is achieved largely by DNA topoisomerases with opposing enzymatic activities. DNA gyrase, encoded by *gyrA* and *gyrB*, introduces negative supercoils, while two other enzymes, DNA topoisomerase I (*topA)* and DNA topoisomerase IV (*parC* and *parE*), remove excess negative supercoils (Pruss *et al*., 1982; Zechiedrich *et al*., 2000). Although the ability to create *topA* null mutants is a clear indication that topoisomerase I is not required for survival (Sternglanz *et al*., 1981; Stupina and Wang, 2005), growth of such mutants often requires compensatory mutations that reduce negative supercoiling (DiNardo *et al.*, 1982; Pruss *et al*., 1982). Indeed, very high levels of supercoiling (hypernegative supercoiling, detected as plasmid topoisomers that cannot be resolved by electrophoresis in agarose gels (Pruss, 1985)) can accumulate in *topA* null mutants (Pruss, 1985) and correlate with growth inhibition (Massé and Drolet, 1999a,b).

One major pathway for generating hypernegative supercoiling in *topA* mutants involves R-loop formation coupled to gyrase activity (Massé and Drolet, 1999c). Hypernegative supercoiling is initiated by the accumulation of negative supercoils behind moving RNA polymerase when topoisomerase I is absent (Massé and Drolet, 1999c). These negative supercoils promote re-hybridization of nascent transcripts to the template DNA strand, which leaves the non-template strand unpaired and the DNA relaxed. Relaxed DNA is a substrate for gyrase, which then introduces more supercoils that lead to increased R-loop formation, more DNA relaxation, and increased gyrase action (Drolet *et al*., 1994; Phoenix *et al*., 1997; Drolet, 2006). Removal of RNA-DNA hybrids by overproduction of RNase HI suppresses the accumulation of hypernegative supercoils and the associated growth defect (Drolet *et al*., 1995; Massé and Drolet, 1999c). Growth inhibition and hypernegative supercoiling also correlate with major gene expression defects, as illustrated by the accumulation of truncated RNAs (Baaklini *et al*., 2004; Baaklini *et al*., manuscript submitted). Within this context, our inability to construct double *topA rnhA* null mutants

did not come as a surprise (Massé and Drolet, 1999b; Drolet *et al*., 1995). Indeed, it was predicted that such double mutants would die from toxic levels of hypernegative supercoiling.

In the present work, we constructed *topA rnhA* double mutants that grew when *rnhA* was expressed from a plasmid-borne gene. Unexpectedly, we found that under nonpermissive conditions the DNA of such mutants was relaxed, not hypernegatively supercoiled. Depletion of RNase HI activity in *topA* null mutants triggered a cellular response that inhibited supercoiling by gyrase. Moreover, the growth inhibition of *topA rnhA* null double mutants correlated with phenotypes reminiscent of segregation defects: extensive cell filamentation with abnormal nucleoid structures and accumulation of anucleate cells. Overproduction of topoisomerase III (*topB*), a type IA enzyme like topoisomerase I, corrected the segregation defects and allowed *topA rnhA* double mutants to grow without restoring supercoiling. Such activity was consistent with topoisomerase III being able to decatenate daughter DNA molecules (Hiasa *et al*., 1994) and use R-loops as a substrate (Broccoli *et al*., 2000). Thus high-level expression of a plasmid-borne *topB* gene provided a second way to allow conditional growth of *topA rnhA* double mutants. Below we describe measurements of bacterial DNA supercoiling and cell morphology that reveal an important role for RNase HI in preventing transcription from perturbing DNA topology beyond viable bounds.

# Results

**Absence of RNase HI prevents accumulation of hypernegative supercoiling associated with a *topA* deficiency.**

We previously described the effects of topoisomerase I defects in living cells using a set of *E. coli* strains in which a temperature-sensitive gyrase mutation allows the growth of *topA* null cells at 37ºC but not at 28ºC owing to reactivation of gyrase (Drolet *et al*., 1995). In such strains hypernegatively supercoiled DNA accumulates following the temperature downshift unless RNase HI is overproduced (Massé and Drolet, 1999c). Overproduction of RNase HI at 28ºC also restores growth. To better understand relationships among hypernegative supercoiling, R-loops, and growth inhibition, we constructed a conditional *topA rnhA* double mutant (transduction was used to introduce an *rnhA::cam* allele into a *topA20*::Tn*10 gyrB*(Ts) strain carrying a plasmid expressing *rnhA* under control of the arabinose-inducible $P_{BAD}$ promoter). Transductants were obtained by plating at 37ºC in the presence but not in the absence of arabinose (not shown). When these transductants were transferred to liquid medium lacking arabinose, cells grew slowly, cell filamentation occurred, and culture growth stopped prematurely, irrespective of temperature (not shown). Thus, when RNase HI is absent, the growth defects of *topA* null *gyrB*(Ts) mutants were seen even at 37ºC. Since colonies arose after plating on solid medium containing arabinose, we conclude that deleting *rnhA* in *topA* mutants is bacteriostatic, not bactericidal (not shown).

Supercoiling was measured with plasmid pGB2*nusB::kan*, a low copy-number derivative of pSC101 that is prone to R-loop-dependent hypernegative supercoiling in *topA* null mutants (Broccoli *et al*., 2004). Plasmid topoisomers were resolved using agarose gel electrophoresis in the presence of 7.5 μg/ml of chloroquine, as previously described (Massé and Drolet, 1999c). Under this condition the more relaxed topoisomers migrated faster; hypernegatively supercoiled DNA also migrated rapidly. With a *gyrB*(Ts) control strain lacking only *topA* (strain PS152), hypernegatively supercoiled DNA accumulated after transfer of cells from 37 to 28ºC (Fig. 1a, lane 2, indicated by [--]). When an *rnhA* null mutation was added (strain PS151, which also carried an inducible *rnhA* gene), and mutant

cells from arabinose-containing agar were incubated overnight at 37ºC in the absence of arabinose, followed by 1:1000 dilution into medium lacking arabinose, cell growth was sufficient for examination of plasmid supercoiling. A portion of the DNA was more relaxed at 37ºC than in *topA* single mutant cells (Fig. 1a, compare lanes 1 and 3), and hypernegatively supercoiled DNA failed to accumulate following a transfer to 28ºC (Fig. 1a, lane 4). The relaxed portion of topoisomers remained extensively relaxed following the temperature downshift (Fig. 1a, lanes 3 and 4, indicated by [rel]). These extensively relaxed topoisomers were not observed in wild-type (lanes 5 and 6) or in *gyrB*(Ts) (lanes 7 and 8) cells. Thus, removal of *rnhA* from a *topA*-deficient strain caused DNA relaxation and loss of hypernegative supercoiling.

When arabinose was added to the diluted (1:1000) overnight culture of the *topA rnhA* double mutant (strain PS151) to produce RNase HI from a plasmid-borne gene, supercoiling was restored at 37ºC (Fig. 1a, compare lane 3, no arabinose, with lane 9, 0.05% arabinose) and accumulation of hypernegatively supercoiled DNA occurred following a temperature downshift (Fig. 1a, lane 10). Since RNase HI was overproduced in this experiment, *topA* null cells accumulated lower amounts of hypernegatively supercoiled DNA than observed with *topA* null cells having only wild-type levels of RNase HI (Fig. 1a, compare lanes 2, *topA* null cells, and 10, *topA rnhA* double mutant with 0.05% arabinose).

Two-dimensional gel electrophoresis confirmed the presence of hypernegatively supercoiled and extensively relaxed DNA, respectively, in the presence and absence of RNase HI (Fig. 1b; hypernegatively supercoiled DNA is seen at bottom part of the curves at high chloroquine concentration ((right panels, indicated by [--]); extensively relaxed DNA is at the far right part of the curves at lower chloroquine concentration (left and middle panels; lines were traced over the more relaxed topoisomers)). Additional two-dimensional gel analyses showed that the more relaxed topoisomers were not positively supercoiled (data not shown). Thus, removal of RNase HI relaxed DNA of *topA* null cells and prevented the accumulation of hypernegatively supercoiled DNA rather than increasing it.

**Supercoiling deficit in a *topA rnhA* null double mutant after transcription inhibition.**

Excess negative supercoiling in *topA* mutants originates largely from transcription. To determine whether the reduced supercoiling in the *topA rnhA* double mutant is specific to transcription-induced supercoiling, mutant cells (strain PS151) were treated with rifampicin, an inhibitor of RNA polymerase. When rifampicin was added to a culture of *topA* null cells (strain PS152, containing wild-type RNase HI) before the temperature downshift, almost all of the hypernegatively supercoiled topoisomers were lost (Fig. 2, top panels). When RNase HI was absent and most of the DNA was seen as extensively relaxed topoisomers, rifampicin had little effect (Fig. 2, bottom panels; a line was traced over the more relaxed topoisomers). Thus the extensive relaxation of supercoiling seen in *topA rnhA* double mutants was related to global supercoiling rather than being specifically linked to transcription. However, our results also show that the effect of the *rnhA* deletion on supercoiling following a temperature downshift was much stronger when transcription was allowed (Fig. 2, compare –rif with +rif, + and – RNase HI).

**Levels of topoisomerase IV and gyrase proteins are not altered by deletion of *rnhA* from *topA* null cells.**

Changes in topoisomerase IV and gyrase concentrations were measured to address the possibility that levels of these proteins account for the extensive DNA relaxation associated with depletion of RNase HI activity in *topA* null cells. ParC and ParE were not overproduced in the *topA20*::Tn*10 gyrB*(Ts) mutant when RNase HI was depleted, as assessed by western blot experiments (Fig. 3, compare lanes 5 and 6 with lanes 7 and 8) and northern blot experiments (data not shown). GyrA and GyrB levels also exhibited no significant difference between the presence and absence of RNase HI (Fig. 3, compare lanes 5 and 6 with lanes 7 and 8).

Lack of significant change in ParC, ParE, GyrA and GyrB level was also observed when the *rnhA* gene was inactivated in the *ΔtopA gyrB*(Ts) strain carrying pPH1243 (Fig. 3, lanes 1 to 4). This plasmid, which expressed *topB* (topoisomerase III) from an IPTG-inducible promoter, allowed the recovery of *rnhA::cam* transductants in a Δ*topA* recipient strain, but only when IPTG was present (Fig. 4a and data not shown). Western blot

experiments with a transductant (strain SB383) confirmed that overproduction of topoisomerase III occurred (Fig. 4b). Plasmid pPH1243 also allowed *topA rnhA* double mutants to grow unless cultures were diluted into IPTG-deficient medium, in which case growth eventually stopped during log phase. Thus, introduction of two restrictive conditions, depletion of RNase HI or removal of topoisomerase III overexpression failed to reveal changes in gyrase and topoisomerase IV concentrations that could explain the extensive DNA relaxation observed with *topA rnhA* double mutants.

**Supercoiling in a *topA rnhA* double mutant is not significantly affected by quinolone-mediated inhibition of topoisomerase IV.**

We also considered the possibility that supercoiling by gyrase was reduced. To test this hypothesis, we used the $gyrA^{L83}$ allele to render gyrase resistant to norfloxacin, thereby allowing selective quinolone-mediated inhibition of topoisomerase IV, the only remaining relaxing activity in *topA* mutants (Zechiedrich *et al*., 2000). The $gyrA^{L83}$ allele was introduced into isogenic *ΔtopA gyrB*(Ts) and *ΔtopA gyrB*(Ts) *rnhA::cam* strains carrying pPH1243 to obtain, respectively, strains VU21 and PS160. Dilution into medium lacking IPTG gradually removed suppression of growth defect by topoisomerase III, but allowed enough growth for examination of plasmid supercoiling (as pointed out in a subsequent section, topoisomerase III overexpression has no effect on supercoiling).

Negative supercoiling increased at 37ºC after inhibition of topoisomerase IV by norfloxacin in both the *topA rnhA* and the *topA* mutants following dilution (Fig. 5, compare *topA rnhA*, strain PS160, lanes 1 and 2 with *topA*, strain VU21, lanes 5 and 6). However, a significant proportion of the topoisomers remained relaxed in the *topA rnhA* mutant (Fig. 5, lane 2, indicated by [rel]). Following a shift to 28ºC, the addition of norfloxacin to inhibit topoisomerase IV strongly stimulated the accumulation of hypernegatively supercoiled DNA in the *topA* mutant (Fig. 5, compare lanes 7 and 8, indicated by [--]), whereas it did not appreciably change the supercoiling level in the *topA rnhA* mutant (Fig. 5, compare lanes 3 and 4). Collectively these results show that topoisomerase IV is not responsible for the strong supercoiling deficit in *topA rnhA* double mutants. It is likely that supercoiling by gyrase is impaired when both RNase HI and topoisomerase I are absent.

**GyrI, a gyrase inhibitor, is not involved in supercoiling inhibition found in a *topA rnhA* double mutant.**

GyrI is encoded by *sbmC*, a well-characterized inhibitor of gyrase (Nakanishi *et al*., 1998; Chatterji and Nagaraja, 2002). Since GyrI is part of the SOS regulon (Baquero *et al*., 1995), which is chronically expressed in *rnhA* mutants (Kogoma *et al*., 1993; McCool *et al*., 2004), it is expected to be at elevated levels in *topA rnhA* double mutants. To examine involvement of GyrI in the supercoiling inhibition in *topA rnhA* double mutants, an *sbmC::kan* allele was introduced into isogenic *ΔtopA gyrB*(Ts) and *ΔtopA gyrB*(Ts) *rnhA::cam* strains carrying pPH1243, to obtain, respectively, strains VU64 and VU70. Both extensive DNA relaxation and lack of hypernegative supercoiling, which are characteristic of the *topA rnhA* double mutant, were seen whether or not *sbmC* was present (Fig. 6, compare lanes 1 and 4 with lanes 2 and 5, respectively). The lack of a significant supercoiling effect from the absence of GyrI was also illustrated by comparing supercoiling in strains VU64 (Fig. 6, $rnhA^+$, lanes 3 and 6) and VU70 (Fig. 6, $rnhA^-$, lanes 2 and 5). Thus, GyrI is not responsible for the DNA relaxation seen in *topA rnhA* double mutants. We also examined a *lexA3* mutant in which the SOS regulon is non-inducible. DNA was relaxed, and hypernegative supercoiling was absent in a *lexA3 topA rnhA* mutant (not shown).

**Cell extracts of a *topA rnhA* double mutant exhibit no supercoiling activity and significantly inhibit supercoiling of wild-type cell extracts.**

The restoration of negative supercoiling is slow and requires a significant increase in the population cell density of strain PS151 (*topA rnhA* double mutant carrying pBAD*rnhA*) following the addition of arabinose to produce RNase HI (not shown). This result suggested involvement of one or more stable factors in inhibiting supercoiling in *topA rnhA* double mutants. To test this idea, we prepared extracts of wild-type (strain AQ634), *gyrB*(Ts) (strain MA249), *gyrB*(Ts) *topA* (strain PS152) and *gyrB*(Ts) *topA rnhA* (strain PS151) cells. Extracts from both wild-type and *gyrB*(Ts) strains exhibited supercoiling activity (Fig. 7a), although with the *gyrB*(Ts) strain an endonuclease activity

was also detected (lane 6). Maximum supercoiling activity was seen at 3.5 μg total protein in the reaction mixture for the *gyrB*(Ts) strain (lane 9); the same amount of protein from wild-type cells exhibited more supercoiling activity (compare wild-type, lane 4 with *gyrB* (Ts), lane 9), and a higher supercoiling level was achieved (not shown). Although supercoiling activity was also detected in crude extracts of *topA* null *gyrB*(Ts) cells (Fig. 7b, lanes 7 to 12, top panel), it was significantly lower than in extracts of isogenic *topA*$^+$ *gyrB*(Ts) cells (Fig. 7a, lanes 6 to 10) and was better seen when the gel was probed with a [$^{32}$P]-labelled DNA fragment (Fig 7b, lanes 7 to 12, bottom panel). Even with radioactive probing, supercoiling activity was undetectable in extracts from the *topA rnhA* double mutant grown in the absence of arabinose to deplete RNase HI activity (Fig. 7b, lanes 1 to 6, bottom panel). These results are consistent with crude extracts of *topA rnhA* null cells containing factor(s) that significantly reduce supercoiling activity.

As a further test for factor(s) inhibiting supercoiling, aliquots of extracts from both *topA* and *topA rnhA* double mutant cells were mixed with extracts from wild-type cells. As shown in Fig. 7c, extracts from the *topA rnhA* double mutant significantly inhibited supercoiling activity of wild-type extracts (compare lanes 1 and 5, which used 2.5 μg protein from the wild-type cell extract with, respectively, 5 and 0 μg protein from the *topA rnhA* double mutant extract). In contrast, inhibition of supercoiling activity by the extract from *topA* null cells was weak (Fig. 7c, compare lanes 1 and 6, using 2.5 μg protein from the wild-type cell extract with 5 μg protein from the *topA rnhA* or the *topA* null extracts). Thus, a strong inhibitory activity present in extracts of *topA rnhA* double mutant explains the failure to detect supercoiling activity and may explain, at least in part, the impairment of supercoiling by gyrase in *topA rnhA* double mutants.

**Topoisomerase III overproduction complements the growth defect of a *topA rnhA* double mutant without restoring supercoiling.**

The data described above failed to relate the growth defect of *topA rnhA* double mutants to excess negative supercoiling. Since in *E. coli* topoisomerase III is not involved in the regulation of supercoiling (Zechiedrich *et al.*, 2000; Lopez *et al*., 2005), we expected

DNA of *topA rnhA* double mutants to remain relaxed during suppression of growth defect by overexpression of topoisomerase III. To test this hypothesis we made use of the finding that in a *topA* null mutant hypernegative supercoiling of pPH1243 is stimulated both by transcription from the strong P*trc* promoter, which is activated by the addition of IPTG, and by translation inhibitors (Broccoli *et al*., 2004; Broccoli and Drolet, unpublished results; spectinomycin was added 15 min before shifting from 37 to 28$^{o}$C). In one experimental set, IPTG was added throughout growth at 37$^{o}$C to overexpress topoisomerase III; in the second set, IPTG was added after spectinomycin so that the transcription effect of IPTG on pPH1243 supercoiling could be observed in the absence of excess topoisomerase III. Fig. 8 shows that pPH1243 exhibited hypernegative supercoiling upon adding IPTG, either before (lane 2) or after (lane 6) the addition of spectinomycin to the *topA* null mutant. In contrast, relaxation of pPH1243 was observed whether or not topoisomerase III was overproduced and irrespective of the presence of spectinomycin in the *topA rnhA* double mutant (Fig. 8, lanes 3, 4, 7 and, 8). Two-dimensional gel electrophoresis confirmed relaxation of pPH1243 (data not shown). As expected, supercoiling was restored in this *topA rnhA* double mutant following the introduction of a plasmid carrying the *rnhA* gene (data not shown), indicating that relaxation is reversible and due to the absence of RNase HI. Collectively, these data support the assertion that overexpression of topoisomerase III suppresses the growth defect of *topA rnhA* double mutants without restoring supercoiling.

**Cells of a *topA rnhA* double mutant form long filaments packed with unevenly distributed DNA.**

Despite the presence of a mechanism to prevent hypernegative supercoiling, *topA rnhA* double mutants do not grow without suppression. This result suggests the presence of supercoiling-independent mechanism(s) by which the absence of RNase HI dramatically perturbs cell physiology. As shown above, the growth defect of a *topA rnhA* double mutant can be rescued by overproducing topoisomerase III (Fig. 4), which can allow chromosome segregation by removing precatenanes during replication (Nurse *et al*., 2003). We therefore

examined the possibility that the growth inhibition of *topA rnhA* double mutants might be related to segregation defects.

Cells were stained with DAPI and prepared for fluorescence microscopy such that both cell morphology and DNA content could be examined. In the absence of topoisomerase III overproduction, cells of the *topA rnhA* double mutant (strain SB383) formed long filaments full of DNA, and nucleoid structures were altered (Fig. 9a, panel 2). Anucleate cells also accumulated (Fig. 9b, yellow arrows), as did cells with very low amounts of chromosomal DNA (Fig. 9b, green arrows). Formation of such cells could result from septum closure on nucleoids, a typical manifestation of partition defects (guillotine effect; Niki *et al*., 1991). Clearly, the *topA rnhA* double mutant fails to produce a significant number of normal cells. However, more than half of the cells containing aberrant nucleoid structures likely remain viable, as cells of strain SB383 incubated in liquid medium lacking IPTG form colonies when plated on solid media containing IPTG to overproduce topoisomerase III (data not shown). Overproduction of topoisomerase III reduced both cell filamentation and DNA content, and it caused DNA to be more regularly distributed within the cells (Fig. 9a, panel 1). Topoisomerase III overproduction also reduced the number of anucleate cells by more than half. Segregation defects were also apparent in the *topA20*::Tn*10 rnhA::cam gyrB*(Ts) mutant carrying pBAD*rnhA* (strain PS151; Fig. 9a, panel 4, -ara); they were corrected by the addition of arabinose to produce RNase HI (panel 3, +ara). Moreover, cells of single *rnhA* and *topA* null mutants failed to show major morphological or nucleoid defects (Fig. 9c, panels 2 and 3 respectively). Thus, combinations of *topA* and *rnhA* null mutations lead to phenotypes reminiscent of segregation defects, thereby explaining the growth inhibition phenotype of *topA rnhA* double mutants.

# Discussion

The work described above, which focused on the properties of *topA rnhA* double mutants, revealed new features of how DNA topology is regulated in bacterial cells. One level concerns DNA supercoiling. Basal supercoiling, which is defined as supercoiling in the absence of transcription (Drlica *et al*., 1988), is set largely by the supercoiling activity of gyrase opposed by the relaxing activities of topoisomerase I and topoisomerase IV. Transcription, through the generation of R-loops coupled with gyrase activity, produces hypernegative supercoiling that is countered by topoisomerase I and RNase HI. The growth defect associated with a deficiency of topoisomerase I is suppressed by partially defective gyrase, overexpression of topoisomerase IV, or overexpression of RNase HI. Surprisingly, *topA rnhA* null double mutants not only lacked hypernegative supercoiling but also contained DNA that was relaxed (Fig. 1 and 8). Evidence was found for the interesting possibility that RNase HI normally regulates factor(s) impairing supercoiling by gyrase. A second level of topology control concerns decatenation of replicated chromosomes. Decatenation is largely a function of topoisomerase IV (Zechiedrich and Cozzarelli, 1995). In our work, a *topA rnhA* double mutant exhibited a defect in chromosome segregation that was corrected by overexpression of topoisomerase III (Fig. 9), an enzyme that has no effect on supercoiling and is capable of decatenation both *in vitro* and *in vivo* (Hiasa *et al*., 1994; Nurse *et al*., 2003). Thus RNase HI, either directly or indirectly, influences both basal levels of supercoiling and chromosome segregation as discussed in the following sections.

**Supercoiling in *topA rnhA* double mutants.**

Previous work showed that gyrase-mediated hypernegative supercoiling occurs during transcription *in vitro* in the absence of RNase HI (Drolet *et al*., 1994; Phoenix *et al.*, 1997) and in *topA* null mutants when RNase HI is not overproduced (Massé and Drolet, 1999c). We expected removal of *rnhA* from *topA* mutants to lead to extensive hypernegative supercoiling, since cells lacking both topoisomerase I and RNase HI cannot be constructed (Massé and Drolet, 1999c; Drolet *et al*., 1995). Unexpectedly, hypernegative supercoiling failed to accumulate in *topA rnhA* double mutants that had been constructed by

conditional expression of *rnhA* or *topB*. However, DNA of such mutants was more relaxed than usual. Relaxation was not an indirect consequence of cell death, because deleting *rnhA* in *topA* null mutants was bacteriostatic, not bactericidal. When arabinose was added to non-growing *topA rnhA* null cells carrying pBAD*rnhA* to induce the synthesis of RNase HI, both growth and supercoiling were restored. More importantly, DNA remained extensively relaxed in a *topA rnhA* double mutant when cell growth was restored by overproducing topoisomerase III. Only when a plasmid carrying *rnhA* was introduced into this mutant that supercoiling was also restored. Therefore, DNA relaxation correlated with depletion of RNase HI activity in *topA* mutants.

Since supercoiling level is set primarily by opposing topoisomerase activities, we considered the possibility that the supercoiling deficit in *topA rnhA* null double mutants was due either to an excess of DNA relaxation activity by topoisomerases or to a loss of gyrase supercoiling activity. One well established mechanism by which excess negative supercoiling is prevented in *topA* mutants is the overproduction of topoisomerase IV (Kato *et al*., 1990; Free and Dorman, 1994). However, western blot experiments showed that topoisomerase IV is not overproduced in *topA* null cells lacking RNase HI activity. Moreover, inhibition of topoisomerase IV by norfloxacin failed to significantly raise supercoiling in a *topA rnhA* double mutant carrying the $gyrA^{L83}$ quinolone-resistance allele. Thus topoisomerase IV was not responsible for the supercoiling deficit in *topA rnhA* double mutants. In agreement with the results of previous experiments showing that topoisomerase III is not involved in supercoiling regulation in *E. coli* (Zechiedrich *et al*., 2000; Lopez *et al*., 2005), overproducing this enzyme had no effect on supercoiling in either *topA* single or *topA rnhA* double mutants. Therefore, relaxation activity of topoisomerases is unlikely to explain the supercoiling deficit in *topA* null cells depleted of RNase HI activity.

Western blot experiments also demonstrated that levels of gyrase protein were not altered following the depletion of RNase HI activity in *topA* mutants. Two experiments indicated that the supercoiling deficit is related to impairment of supercoiling by gyrase. First, while inhibiting topoisomerase IV by norfloxacin in a single *topA* null mutant strongly promoted hypernegative supercoiling, it did not significantly stimulate supercoiling in a double *topA rnhA* null mutant. Second, supercoiling activity could not be

detected in cell extracts of a *topA rnhA* null double mutant (strain PS151), and these extracts significantly inhibited the supercoiling activity when mixed with extracts from wild-type cells. Thus, the supercoiling deficit in *topA* null cells depleted of RNase HI activity is likely related to a cellular response that leads to the inhibition of gyrase.

The cellular response leading to the impairment of gyrase activity in double *topA rnhA* null mutants is currently unknown. The response is related neither to the SOS regulon, which is constitutively induced in the absence of RNase HI (Kogoma *et al*., 1993; McCool *et al*., 2004), nor to the presence of the gyrase inhibitor, GyrI, a member of the SOS regulon (Baquero *et al*., 1995). Previous results also failed to demonstrate an effect of GyrI on supercoiling *in vivo* (Chatterji *et al*., 2003). While supercoiling by gyrase can be prevented by a direct interaction between the enzyme and a specific inhibitor acting like GyrI, it may also be inhibited by low [ATP]/[ADP] (Westerhoff *et al*., 1998; Drlica, 1992) and indirectly by proteins that interact with DNA, such as the abundant nucleoid proteins Fis, H-NS and HU (Travers and Muskhelishvili, 2005ab). Additional experiments are required to work through the many possible ways in which gyrase activity can be lowered.

**Segregation defect and growth inhibition suppressed by topoisomerase III.**

The growth inhibition associated with *topA rnhA* double mutants correlated with phenotypes seen previously with chromosomal segregation defects: extensive cell filamentation, abnormal nucleoid structures, and accumulation of anucleate cells. Thus, the simultaneous absence of topoisomerase I and RNase HI leads to segregation defects not observed with cells lacking only one of the enzymes. R-loops may be involved, since a relationship between topoisomerase I and RNase HI is well established. If R-loops persist in *topA rnhA* double mutants, particularly at *oriK* sites where constitutive, stable DNA replication is initiated (Kogoma, 1997), excess replication could occur. Over-replication may saturate the segregation capacity of the cell, thus requiring more decatenation activity than can be provided by excess topoisomerase III.

Unregulated over-replication is also known to lead to collapse of replication forks, DNA double-strand breaks (Kouziminova *et al*., 2004; Simmons *et al*., 2004; Michel *et al.*, 2007), and ultimately hyper-recombination, which can cause segregation defects (Lopez *et*

*al*., 2005; Zhu *et al*., 2001; Zahradka *et al*., 1999; Magner *et al*., 2007). Accumulation of unresolved recombination intermediates interferes with chromosome segregation; resolution of these structures can be performed by topoisomerase III (Lopez *et al*., 2005; Zhu *et al*., 2001), even when it is present at normal or very low levels (less than 10 copies per cell; Digate and Marians, 1989).

The extensive DNA relaxation in *topA rnhA* double mutants could also contribute to segregation defects, since the inhibitory effect of temperature-sensitive gyrase mutations on segregation (Steck and Drlica, 1984) is thought to be due to DNA relaxation that then suppresses decatenation by topoisomerase IV (Zechiedrich *et al*., 1997; Holmes and Cozzarelli, 2000). Overproduction of topoisomerase III allows chromosome segregation when topoisomerase IV is inactive (Nurse *et al*., 2003). However, in the context of extensive DNA relaxation, the ability of topoisomerase III to perform decatenation might be reduced because single-stranded DNA regions, the substrate for topoisomerase III, are expected to be infrequent. Such regions can also be provided by R-loops (Broccoli *et al*., 2000).

**Biological function of RNase HI**

The first function attributed to RNase HI in *E. coli* was a role in the removal of RNA primers of Okazaki fragments (Funnell *et al.*, 1986; Ogawa and Okazaki, 1984). However, this enzyme cannot remove the last ribonucleotides at the RNA-DNA junctions. In fact, 5'-3' exonuclease activity (e.g. polymerase I) plays the major role in the removal of RNA primers. Interestingly, RNase H activity in various bacterial species was recently shown to be dispensable for complete RNA primer removal (Fukushima *et al*., 2007). Only 5'-3' exonuclease activity was shown to be indispensable for this process. Based on the results presented in the present work we propose that a major function of RNase HI, and possibly other bacterial RNase H molecules, involves the control of DNA topology via R-loops.

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# Experimental procedures

***E. coli* strains.** *Escherichia coli* strains used in this work are described in Table 1. Strains were constructed by transduction with phage P1*vir* as previously described (Miller, 1992). When needed, tetracycline (10 μg/ml), chloramphenicol (15 μg/ml) or kanamycin (50 μg/ml) was added to the medium. PCR was used to confirm the presence of only the *rnhA::cam* allele and the *sbmC::kan* allele, respectively, on the chromosome of the *rnhA* null and the *sbmC* null transductants.

**Plasmids.** pBAD18*rnhA* was constructed by placing an EcoRI-HindIII fragment carrying the *rnhA* gene under the control of the $P_{BAD}$ promoter of pBAD18 (Guzman *et al*., 1995). The *rnhA* gene was obtained by PCR from pSK760 (Drolet *et al*., 1995) by using d(GTCAGAATTCCAGGAAGTCTACCAGA) and d(GTCAAAGCTTGGCAATGTCGTAAACC) oligonucleotides. pGB2*nusB::kan* is a pSC101 derivative that was constructed by inserting the pUC4K EcoRI fragment carrying a kanamycin-resistance cassette into the ScaI site of pGB2nusB (Friedman *et al.*, 1990). pPH1243 is a pTrc99a derivative carrying the *topB* gene under the control of the IPTG-inducible P*trc* promoter (Broccoli *et al*., 2000).

**Plasmid extraction for supercoiling analysis**. Cells were grown overnight at 37°C in LB medium supplemented with cysteine (50 μg/ml, for the RFM475 derivatives), thymine (10 μg/ml, for the MA251 derivatives) or tryptophan (50 μg/ml, for VU35 and VU95 strains). When required, ampicillin (50 μg/ml), spectinomycin (30 μg/ml), arabinose (0.05%) or IPTG (1 mM) were added. Overnight cultures diluted in pre-warmed medium (37°C), were grown to an $OD_{600}$ of ~0.5 at which time an aliquot of cells was recovered for plasmid extraction while the remaining culture was transferred to 28°C. Aliquots of cells were recovered for plasmid extraction at the indicated times. Growth was arrested by transferring cells into a tube filled with ice, thus immediately lowering the temperature of the culture to

0°C. Plasmid DNAs were extracted by alkaline lysis as previously described (24). For experiments using derivatives of MA251, overnight cultures were diluted 1:1000; they were diluted to an $OD_{600}$ of 0.03 for the experiments using SB383, VU21, PS160, VU64, and VU70.

**Plasmid topoisomer analysis.** One-dimensional and two-dimensional agarose gel electrophoresis in the presence of chloroquine was performed in 0.5 X TBE as described (Massé *et al*., 1997). After electrophoresis, the gels were dried and prepared for *in situ* hybridization with random prime-labelled probes as described (Massé *et al*., 1997). Images were obtained by using a Phosphorimager Typhoon 9400 (Amersham Biosciences).

**Western blot analysis**. The equivalent of 200 µl of cell culture at an $OD_{600}$ of 0.7 was used for Western blot analysis. The cell pellets were lysed by boiling in sodium dodecyl sulphate (SDS). The proteins were separated by SDS-polyacrylamide (7.5 %) gel electrophoresis. Western blots were performed as described previously (Sambrook *et al*.,1989) by using nitrocellulose membranes (Hybond-ECL, GE Healthcare). After transfer, the membranes were stained with Ponceau S (Fisher Scientific) to confirm that similar amounts of protein were loaded in each lane. ParC and ParE antibodies were obtained from Dr Kenneth J. Marians (Memorial Sloan-Kettering Cancer Center, New York, New York). GyrA and GyrB antibodies were purchased from John Innes Enterprises Ltd (John Innes Centre, Norwich Research Park Colney, Norwich, UK). TopB antibodies were obtained from Dr Russell DiGate (Department of Pharmaceutical Sciences, Philadelphia College of Pharmacy, Philadelphia, PA). The ECL Plus detection kit (GE Healthcare) was used to reveal the specific proteins.

**Gyrase assays in crude cell extracts.** Cells were grown in LB medium at $37^0$C to an $OD_{600}$ of 0.7 and transferred to $28^0$C for 30 minutes. Cells were recovered and prepared for gyrase assays in crude extracts as previously described (DiNardo *et al*., 1982). 0.2 µg of relaxed pBR322 DNA (prepared by using wheat germ topoisomerase I from Sigma-Aldrich) was used in the assays.

**Microscopy.** RFM443, RFM475, PH379 and SB383 cells were grown overnight on LB plates supplemented, when required, with cysteine (50 μg/ml), ampicillin (50 μg/ml), chloramphenicol (10 μg/ml) and/or tetracycline (10 μg/ml). When needed, IPTG (1 mM) was added to the plates of SB383 cells. PS151 cells were grown overnight on LB plates supplemented with thymine (50 μg/ml) and, when needed, arabinose (0.05%). The plates were incubated at 37°C. After overnight growth, cells were resuspended in pre-warmed (37°C) liquid LB medium (supplemented as requested) to obtain a starting $OD_{600}$ of about 0.01. Cells were grown at 37°C to an $OD_{600}$ of 0.8. 150 μl of cells were harvested, centrifuged, and resuspended in 77% ethanol (fixing solution). The cells were washed with 500 μl of 0.9% NaCl, centrifuged and resuspended in 100 μl of 0.9% NaCl. 3 μl of the fixed samples were spread on slides pre-treated with a Poly-L-Lysine solution (Sigma) and allowed to air dry at room temperature. 5 μl of slow fade gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole; from Invitrogen) was deposited on the slides and sealed with a cover glass. Fluorescence pictures were obtained with a Nikon E600 equipped with a 100-W mercury lamp and standard DAPI filters using the X100 oil immersion objective. The images were captured on the computer using the Nikon ACT-1 software. Exposure time was 1/50s at maximum sensitivity. For phase contrast microscopy, the microscope was adjusted to the phase contrast optical system and pictures were taken at X100 oil immersion objective with the Ph3 annulus. Exposure time was 1/120s at normal sensitivity. The images were processed with Adobe Photoshop.

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# TABLES AND FIGURES

**Table 1. *Escherichia coli* strains used in this study.**

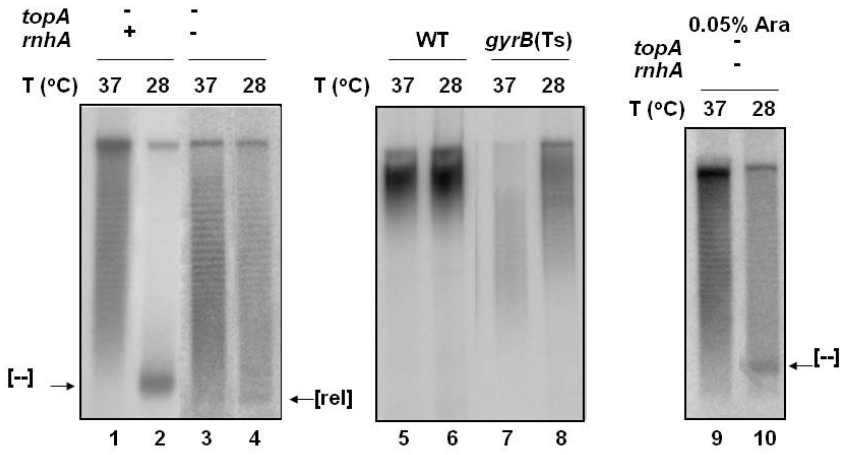
| Strain | Genotype | Source or Ref. |
|---|---|---|
| AQ634 | *ilv metB his-29 trpA9605 pro thyA deoB* (or *C*) | Hraiky *et al*., 2000 |
| MA249 | AQ634 *gyrB221*(cou$^R$) *gyrB203*(Ts) *zie-3163*::Tn*10kan* | Hraiky *et al*., 2000 |
| MA251 | MA249 *gyrB221*(cou$^R$) *gyrB203*(Ts) *zie-3163*::Tn*10kan topA20*::Tn*10* | Hraiky *et al*., 2000 |
| CM23 | MA251 pBAD18*rnhA* | This work |
| RFM430 | *rpsL, galK2, ΔtrpE* | Drolet *et al*., 1995 |
| MIC1020 | AB1157 *rnhA::cam* | Itaya and Crouch, 1991 |
| PH379 | RFM430 *rnhA::cam* | This work |
| PS147 | CM23 *rnhA::cam* | This work |
| PS151 | PS147 pGB2*nusB::kan* | This work |

| | | |
|---|---|---|
| PS152 | MA251 pGB2*nusB::kan* | This work |
| RFM445 | *rpsL galK2 gyrB221*(cou$^R$) *gyrB203*(Ts) Δ*lac74* | Drolet *et al*., 1995 |
| RFM475 | *rpsL galK2 gyrB221*(cou$^R$) *gyrB203*(Ts) *Δ(topA cysB)204* Δ*lac74* | Drolet *et al*., 1995 |
| SB224 | RFM475 pPH1243 | This work |
| SB383 | SB224 *rnhA::cam* | This work |
| LZ1 | Hfr *thi gyrA*$^{L83}$ *zei-723*::Tn*10* | Zechiedrich *et al*., 2000 |
| PS158 | RFM475 *gyrA*$^{L83}$ *zei-723*::Tn*10* | This work |
| VU21 | PS158 pPH1243 | This work |
| PS160 | SB383 *gyrA*$^{L83}$ *zei-723*::Tn*10* | This work |
| VU48 | MG1655 *sbmC::kan* | Carmen Gomez-Eichelmann |
| VU56 | RFM475 *sbmC::kan* | This work |
| VU64 | VU56 pPH1243 | This work |

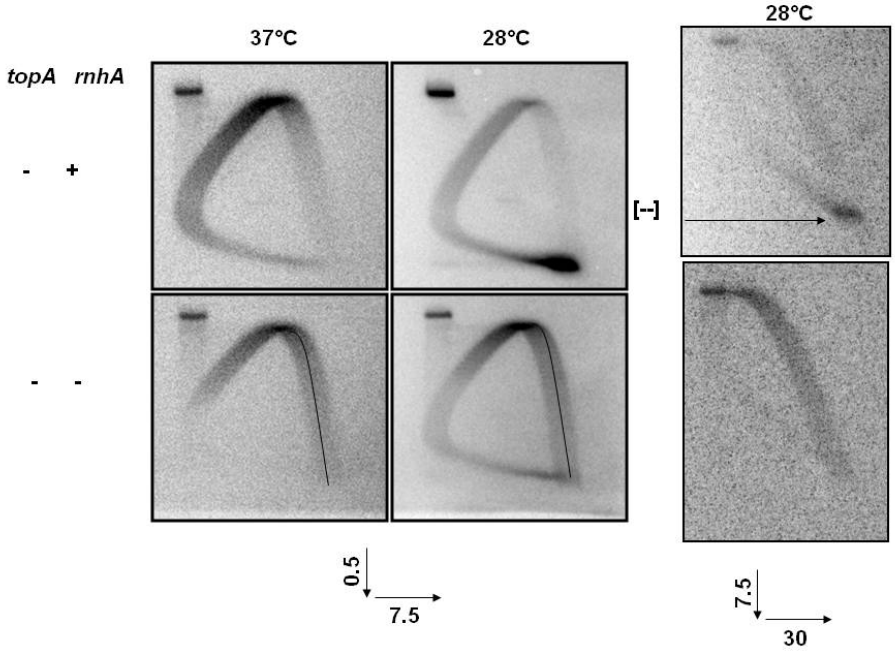
| | | |
|---|---|---|
| VU70 | VU64 *rnhA::cam* | This work |

**Figure 1. Supercoiling in a *topA rnhA* null double mutant.** AQ634 (wild-type), MA249 (*gyrB* (Ts)), PS152 (*topA*$^{-}$, *rnhA*$^{+}$/pGB2nusB::kan) and PS151 (*topA*$^{-}$, *rnhA*$^{-}$ /pGB2nusB::kan and pBAD18*rnhA*) cells were grown overnight at $37^{0}$C in the absence of arabinose. Overnight cultures were diluted 1:1000 in LB medium with or without arabinose (0.05%) as indicated and grown at $37^{0}$C. DNA was extracted just before the temperature downshift and 60 minutes after the downshift (28$^{o}$C). DNA samples were loaded on agarose gels for 1-D (with 7.5 μg/ml of chloroquine in a) or 2-D (chloroquine concentrations in μg/ml are indicated by arrows in b) electrophoresis. The gels were hybridized with a probe to detect pGB2nusB::kan. [--] points to hypernegatively supercoiled DNA. [rel] indicates extensively relaxed DNA. In b), lines were traced over the more relaxed topoisomers.

A


topA
rnhA
T (°C) 37 28 37 28
[--]
[rel]
1 2 3 4
WT
gyrB(Ts)
T (°C) 37 28 37 28
5 6 7 8
0.05% Ara
topA
rnhA
T (°C) 37 28
[--]
9 10

B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28°C
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- +
- -
[--]
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7.5
7.5
30

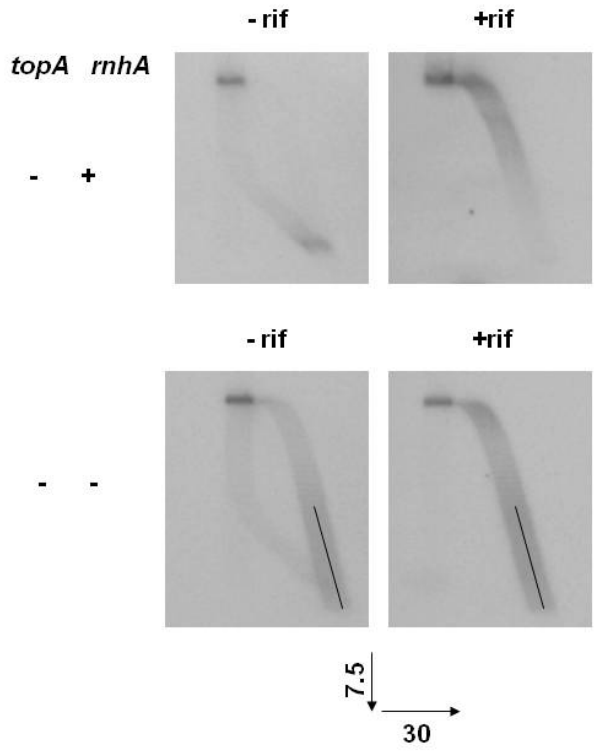



**Figure 2. Supercoiling in a *topA rnhA* null double mutant after transcription inhibition.** PS152 (*topA*$^-$, *rnhA*$^+$/ pGB2nusB::kan) and PS151 (*topA*$^-$, *rnhA*$^-$/ pGB2nusB::kan and pBAD18*rnhA*) cells were grown at 37ºC in the absence of arabinose as indicated in the legend to Fig. 1. Rifampicin (250 μg/ml) was added or not as indicated, 15 minutes before the temperature downshift and the DNA was extracted 60 min after the downshift (28ºC). DNA samples were loaded on an agarose gel for 2-D electrophoresis (chloroquine concentrations in μg/ml are indicated by arrows). The gel was hybridized with a probe to detect pGB2nusB::kan. Lines were traced over the more relaxed topoisomers (bottom panels).

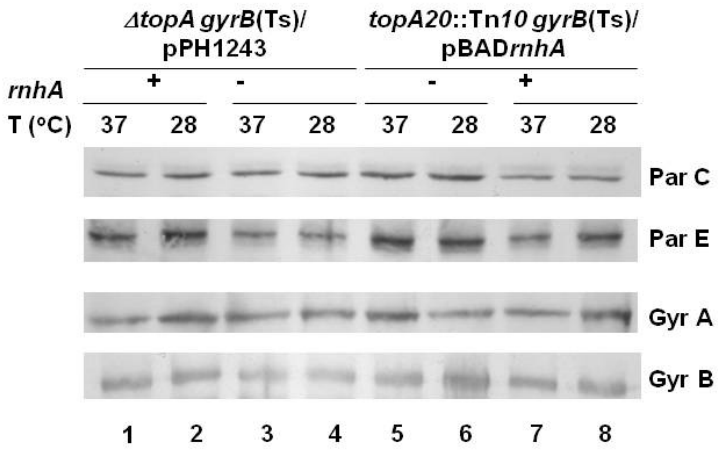



**Figure 3. Western blot analysis to measure topoisomerase IV and gyrase levels in *topA rnhA* mutants.** RFM475 (*gyrB*(Ts) *ΔtopA*), SB383 (*gyrB*(Ts) *ΔtopA rnhA::cam*/pPH1243), PS147 (*gyrB*(Ts) *topA20*::Tn*10 rnhA::cam*/pBAD18*rnhA*) and MA251 (*gyrB*(Ts) *topA20*::Tn*10*) cells were grown at 37°C in LB medium (no IPTG, no arabinose). Aliquots of cells were used for western blots as described in Experimental procedures. Lanes 1, 3, 5 and 7, aliquots of cells grown at 37°C; lanes 2, 4, 6 and 8, aliquots of cells exposed to 28°C for one hour.

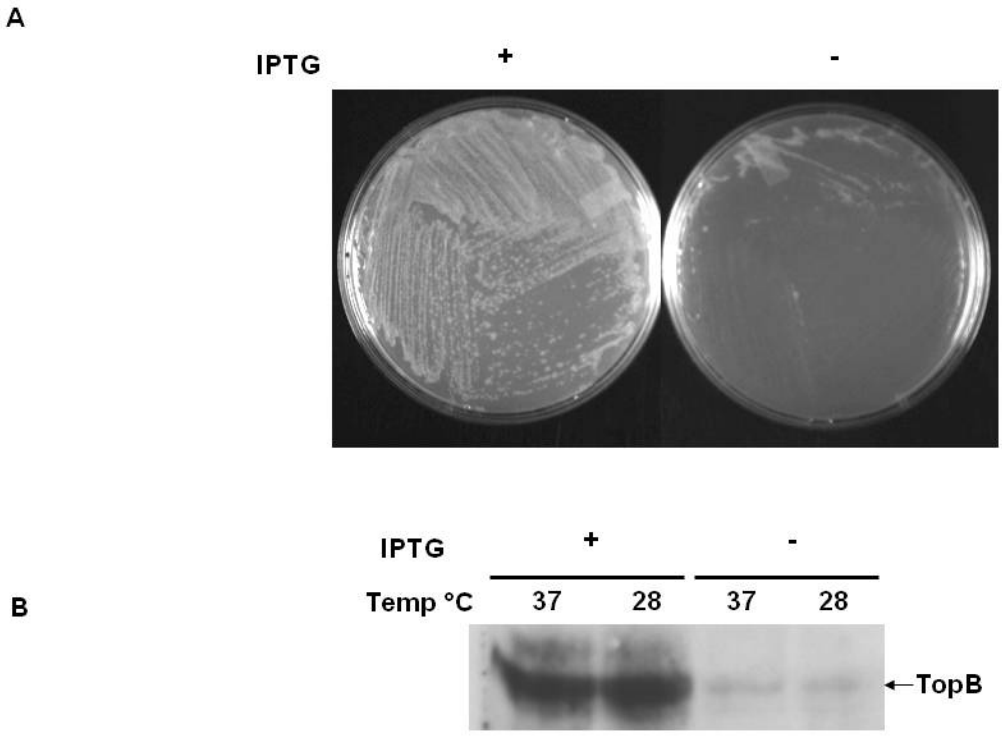



**Figure 4. Complementation of a *topA rnhA* mutant by topoisomerase III overproduction.** In (a) SB383 (*gyrB*(Ts) *ΔtopA rnhA::cam*/pPH1243) cells were streaked on solid LB medium with 1 mM or no IPTG as indicated. The plates were incubated overnight at 37ºC. In (b) SB383 cells were grown in LB at 37ºC with or without 1 mM IPTG as indicated and aliquots were used for western blots with anti-topoisomerase III antibodies. Aliquots were also obtained from cells 60 min after a temperature downshift to 28ºC.

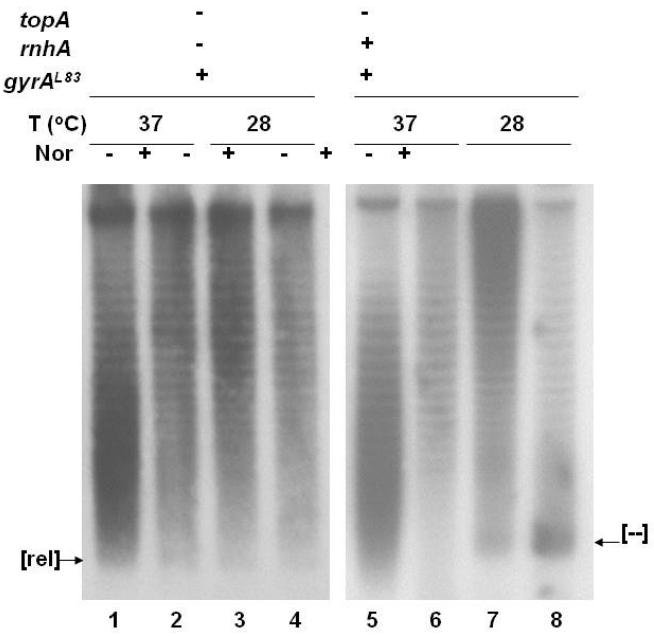



**Figure 5. Topoisomerase IV inhibition does not promote hypernegative supercoiling in a double *topA rnhA* mutant.** PS160 (*topA*$^-$, *rnhA*$^-$ *gyrA*$^{L83}$/pPH1243) and VU21 (*topA*$^-$, *rnhA*$^+$ *gyrA*$^{L83}$/pPH1243) cells were grown at 37ºC in LB without IPTG to log phase and norfloxacin (60 µM final) was added or not as indicated. Aliquots of cells were taken for DNA extraction 30 min later and the remaining cells were transferred to 28ºC. 30 min later aliquots of cells were taken for DNA extraction. DNA was analyzed in an agarose gel with 7.5 µg/ml chloroquine. The gel was hybridized with a probe to detect pPH1243 topoisomers. [--] point to hypernegatively supercoiled DNA. [rel] indicates extensively relaxed DNA.

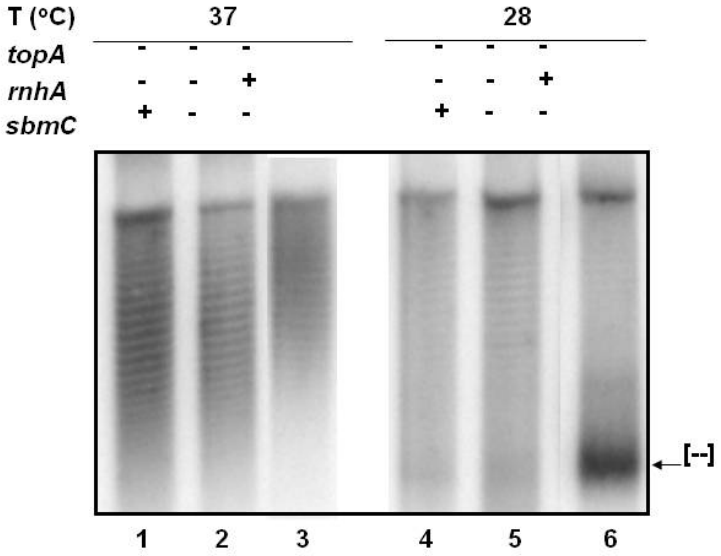
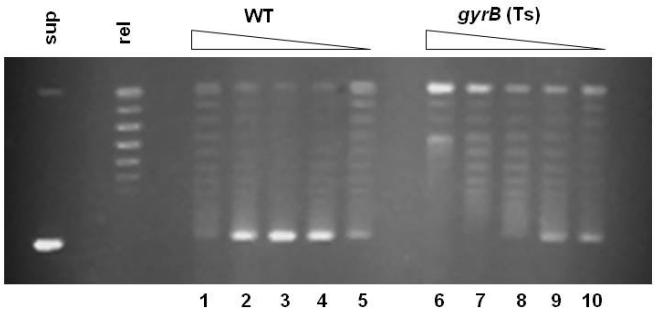



**Figure 6. GyrI, a gyrase inhibitor, plays no role in supercoiling inhibition in a *topA rnhA* null double mutant.** SB383 (*topA*$^-$, *rnhA*$^-$, *sbmC*$^+$/pPH1243), VU70 (*topA*$^-$, *rnhA*$^-$, *sbmC*$^-$/pPH1243) and VU64 (*topA*$^-$, *rnhA*$^+$, *sbmC*$^-$/pPH1243) cells were grown at 37ºC in the presence of 1 mM ITPG. Spectinomycin was added to 400 µg/ml 15 min before the temperature downshift to 28°C. DNA was extracted 60 min after the downshift and samples were loaded on an agarose gel (with 7.5 µg/ml of chloroquine) for 1-D electrophoresis. The gel was hybridized with a probe to detect pPH1243 topoisomers. [--] point to hypernegatively supercoiled DNA.

**Figure 7. Supercoiling assays in crude cell extracts.** Crude cell extracts were prepared and used for supercoiling assays as described in Experimental procedures. In (a), the amounts of protein used in the assays were 28 μg (lanes 1 and 6), 14 μg (lanes 2 and 7), 7 μg (lanes 3 and 8), 3.5 μg (lanes 4 and 9) and 1.75 μg (lanes 5 and 10). The reactions were incubated at 30ºC for 2 hours. sup is supercoiled pBR322 and rel is relaxed pBR322. WT and *gyrB*(Ts) respectively indicate AQ634 and MA249 strains that were used in the experiment. In (b), the amounts of protein used in the assays were 40 μg (lanes 1 and 7), 20 μg (lanes 2 and 8), 10 μg (lanes 3 and 9), 5 μg (lanes 4 and 10), 2.5 μg (lanes 5 and 11) and 1.25 μg (lanes 6 and 12). The reactions were incubated at 30ºC for 4 hours with twice the amount of ATP. At the top of the ethidium bromide stained gel (top panel), sup is supercoiled pBR322 and rel is relaxed pBR322. The bottom panel is a picture of the gel probed with a random prime-labelled fragment hybridizing to the *bla* gene of pBR322. The strains used in the experiment were PS151 (*topA*$^-$*, rnhA*$^-$/ pGB2nusB::kan and pBAD18*rnhA*) and PS152 (*topA*$^-$*, rnhA*$^+$/ pGB2nusB::kan). They were grown in the absence of arabinose and the log phase cultures were obtained from the overnight ones diluted 1:1000. In (c), 2.5 μg of proteins from a crude cell extract of the wild-type strain (AQ634) were mixed with 0, (lane 5), 5 (lane 1), 2.5 (lane 2), 1.25 (lane 3) or 0.63 μg (lane 4) of proteins from the crude extract of PS151 strain used in (b) and with 5 (lane 6), 2.5 (lane 7), 1.25 (lane 8) and 0.63 μg (lane 9) of proteins from the crude extract of PS152 strain used in (b). The reactions were incubated at 30$^0$C for 2 hours. sup is supercoiled pBR322 and rel is relaxed pBR322.

A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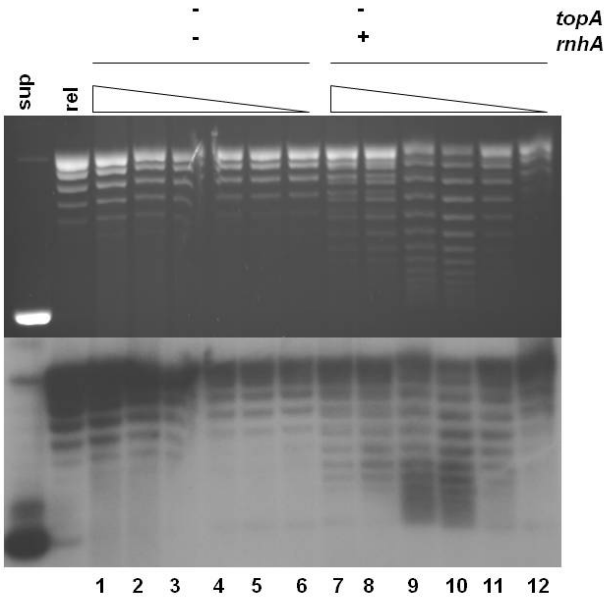
B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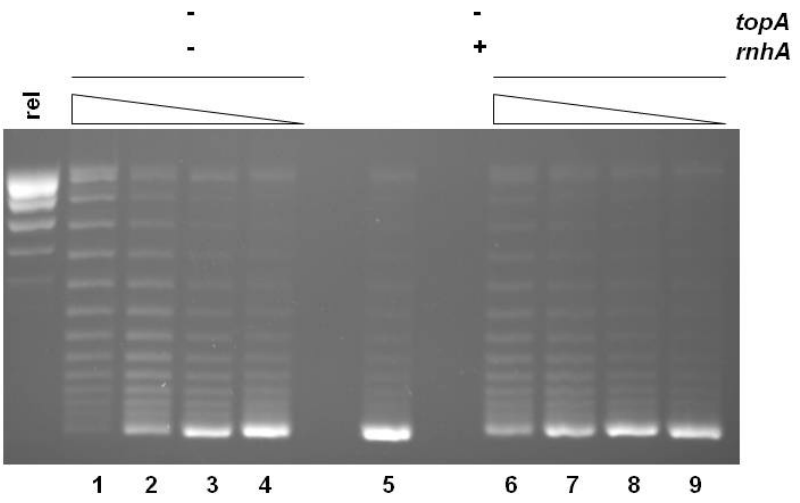


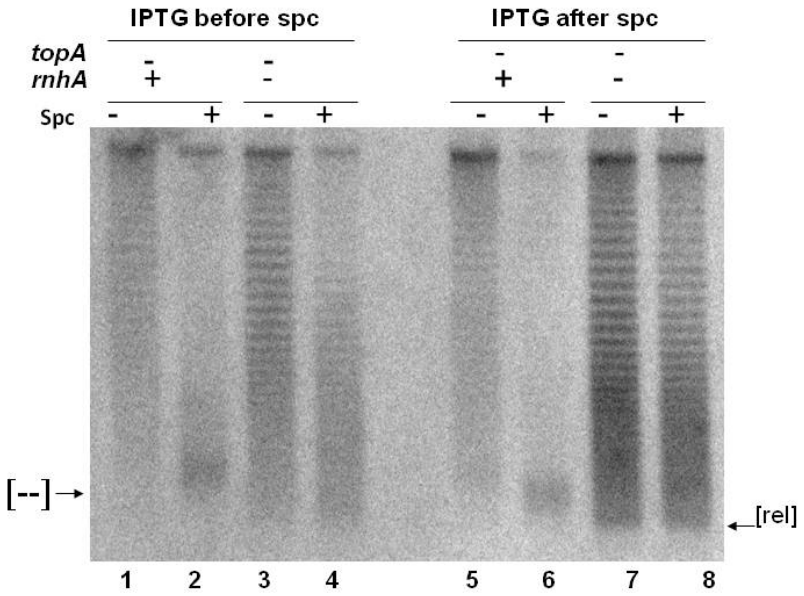
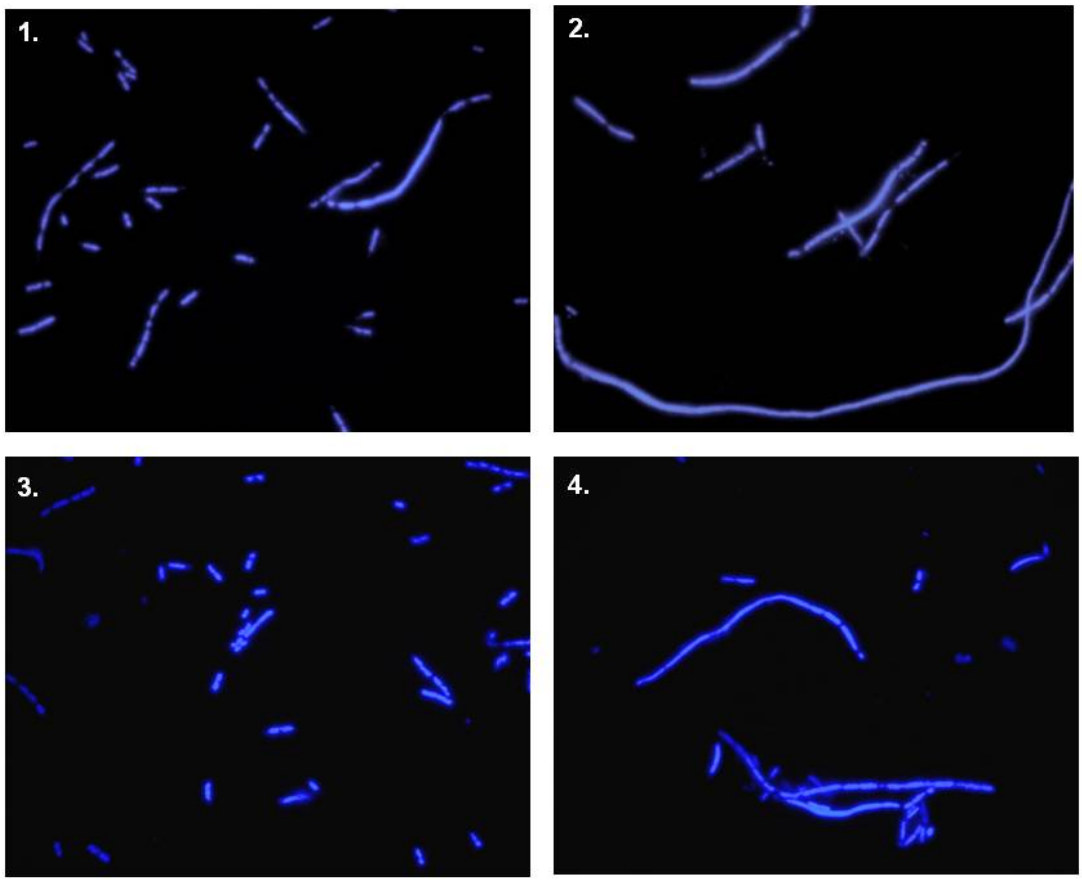



**Figure 8. Topoisomerase III overproduction does not change supercoiling in a *topA rnhA* null double mutant.** SB224 (*topA*$^{-}$, *rnhA*$^{+}$/ pPH1243) and SB383 (*topA*$^{-}$, *rnhA*$^{-}$ /pPH1243) cells were grown in LB to log phase at 37$^{0}$C. Spectinomycin was added to 400 µg/ml 15 min before the temperature downshift to 28$^{o}$C. IPTG before spc means that IPTG was added to 1 mM during the growth at 37 $^{o}$C whereas IPTG after spc means that IPTG was added to 1 mM 5 min after the temperature downshift. DNA was extracted 60 min after the downshift and used for 1-D (7.5 µg/ml chloroquine) gel analysis. The gel was hybridized with a probe to detect pPH1243. [--] points to hypernegatively supercoiled DNA. [rel] indicates extensively relaxed DNA.

**Figure 9. DAPI staining of *topA rnhA* null mutants reveals segregation defects that are corrected by topoisomerase III overproduction.** Cells were grown and prepared for microscopy as described in Experimental procedures. In a), 1 and 2 are fluorescence pictures of DAPI stained SB383 cells (*gyrB*(Ts) *ΔtopA rnhA::cam*/pPH1243) respectively grown in the presence and the absence of IPTG both during overnight growth on plates and during growth in liquid. 3 and 4 are fluorescence pictures of DAPI stained PS151 cells (*gyrB*(Ts) *topA20*::Tn*10 rnhA::cam*/pBAD18*rnhA*) respectively grown in the presence and the absence of arabinose both during overnight growth on plates and during growth in liquid. Superimposed pictures of phase contrast and fluorescence (by using Adobe Photoshop) of DAPI stained SB383 (no IPTG) cells are shown in b). Yellow and green arrows indicate respectively anucleate cells and cells with low DNA content. In c), 1 to 3 are fluorescence pictures of DAPI stained RFM430 (wild-type), PH379 (*rnhA::cam*) and RFM475 (*gyrB*(Ts) *ΔtopA*) cells, respectively.

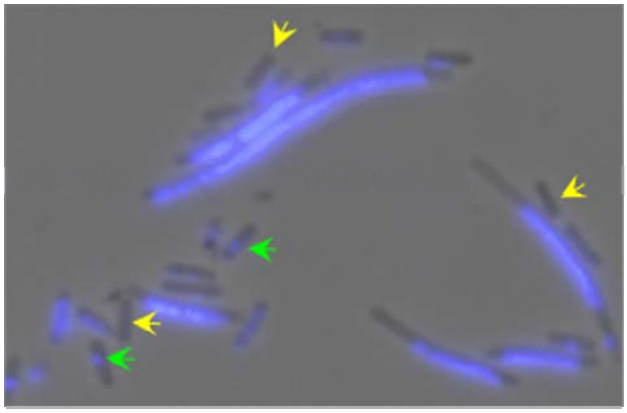
A



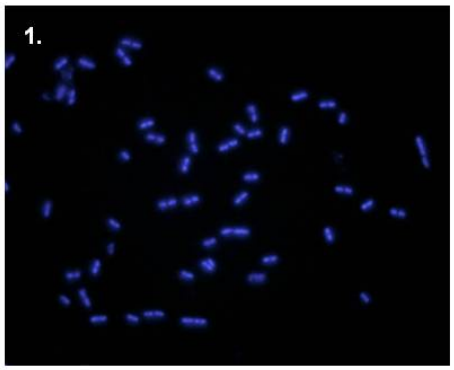
B

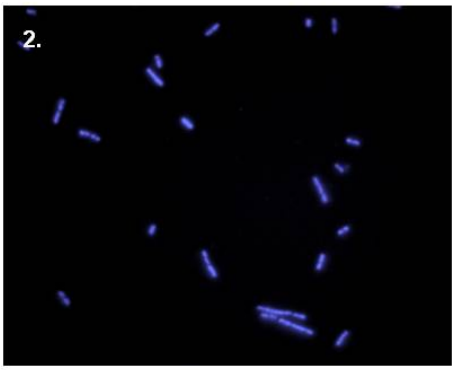
*topA rnhA* null mutant/pPH1243

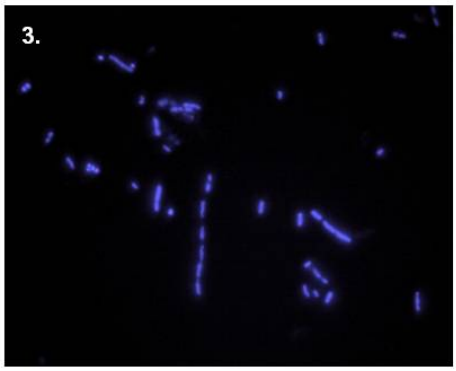
-ITPG



C



# Preface to Chapter 3

In chapter 2, we demonstrated that *topA rnhA gyrB*(Ts) exhibited phenotypes consistent with chromosome segregation defects. These cells formed long filaments full of DNA. The growth and segregation defects of these mutants were corrected by overproducing topoisomerase III, a type IA topoisomerase. This suggested that type IA topoisomerases could play a role in chromosome segregation. Here we investigated the role of type IA topoisomerases in chromosome segregation. To do this, we use genetic approaches coupled with flow cytometry and microscopy to examine cell morphology. We found that both the growth defect and Par-phenotypes of a *gyrB*(Ts) mutant at nonpermissive temperatures were corrected by deleting *topA*, but only when *topB* was present. Our data point to a role for type IA topoisomerases in chromosome segregation when gyrase is inefficient.

**Manuscript II:** **Usongo, V.,** Tanguay, C., Nolent, F., Egbe, J., and Drolet, M. (2013). Interplay between type IA topoisomerases and gyrase in chromosome segregation in *Escherichia coli*. Journal of Bacteriology. **195:**1758-68.

This project was designed by Dr. Marc Drolet. I played a significant role towards the publication of this manuscript. I performed half of the experiments reported in this manuscript. In addition, I prepared the figures, contributed to ideas through informal discussions and proofread the final draft prior to submission.

# CHAPTER 3: Manuscript II

**Interplay between type IA topoisomerases and gyrase in chromosome segregation in *Escherichia coli***

Valentine Usongo, Cynthia Tanguay, Flora Nolent, Jill Egbe, and Marc Drolet

Running title: type 1A topoisomerases in bacterial chromosome segregation

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# Abstract

*Escherichia coli* possesses two type 1A topoisomerases, topo I (*topA*) and topo III (*topB*). Topo I relaxes excess negative supercoiling and *topA* mutants can grow only in the presence of compensatory mechanisms, such as gyrase mutations. *topB* mutants grow as well as wild-type cells. *In vitro* topo III, but not topo I, can efficiently decatenate DNA during replication. However, *in vivo*, a chromosome segregation defect is only seen when both type 1A topos are absent. Here we present experimental evidence for an interplay between gyrase and type 1A topos in chromosome segregation. We found that both the growth defect and the Par- phenotypes of a *gyrB*(Ts) mutant at nonpermissive temperatures were significantly corrected by deleting *topA,* but only when *topB* was present. Overproducing topo IV, the major cellular decatenase, could not substitute for *topB*. We also show that overproducing topo III at a very high level could suppress the Par- phenotype. We have previously found that the growth and chromosome segregation defects of a triple *topA rnhA gyrB*(Ts) mutant in which gyrase supercoiling activity was strongly inhibited, could be corrected by overproducing topo III. We show here that this overproduction could be bypassed by substituting the *gyrB*(Ts) allele for a $gyrB^+$ one or by growing cells in a minimal medium, conditions that reduced both $topA^-$ and $rnhA^-$-dependent unregulated replication. Altogether our data point to a role for topo III in chromosome segregation when gyrase is inefficient and suggest that topo I plays an indirect role via supercoiling regulation.

# Introduction

DNA topoisomerases are ubiquitous enzymes found in eubacteria, archaebacteria and eukaryotes that solve the topological problem associated with replication, transcription and recombination (1). Type 1A topos cleave one DNA strand at a time to change the DNA linking number in single steps, and they all require, to various extent depending on the enzyme, an exposed single-stranded region within the DNA substrate. *E. coli* possesses two type 1A enzymes, namely topo I (*topA*) and topo III (*topB*). Topo I binds to single-stranded DNA regions close to double-stranded ones (2) thus explaining its major role in the relaxation of excess negative supercoiling, that is mostly generated during transcription (3-5). Consistent with this function is the finding that many *topA* mutants can grow owing to the presence of compensatory mutations in *gyrA* or *gyrB* that reduce the supercoiling activity of gyrase (6, 7). One major consequence of excess negative supercoiling is R-loop formation and RNA degradation (5, 8, 9).

Topo III is a very low abundance protein (10) and its cellular role is not well defined. This is largely due to the fact that, as opposed to *topA* null mutants, *topB* null mutants display no obvious phenotype (they grow as well as wild-type strains, at least under standard laboratory conditions (10)). Topo III requires stable single-stranded DNA regions for activity and does not efficiently relax negatively supercoiled DNA (11). In fact, topo III plays no role in supercoiling regulation *in vivo* (12, 13). *In vitro*, topo III is a potent decatenase providing that single-stranded regions are present on the DNA (11).

DNA gyrase, the enzyme responsible for the introduction of negative supercoiling in DNA, plays major roles in replication. Firstly, via negative supercoiling, gyrase facilitates DNA melting at *oriC* in order for replication initiation to take place (14). Secondly, gyrase removes the left-handed positive supercoiling generated in front of moving replication forks (15). In fact, it is believed that most of the intertwining generated by replication is normally eliminated by gyrase (16). The positive supercoiling generated by replication can also migrate behind the replication fork, providing that the replication complex is free to rotate, which leads to the formation of precatenanes (intertwining of the pair of replicated chromosome segments; 17-19) that can be removed by topo IV, the major

cellular decatenase. Once the chromosome is fully replicated, the remaining precatenanes becomes catenanes that are also eliminated by topoisomerase IV, thus allowing chromosome segregation to take place. *In vitro*, topo III alone can support replication presumably by acting on single-stranded DNA at the replication fork to remove precatenanes (20-22). This mechanism has been proposed to explain the suppression of the chromosome segregation defect of topo IV mutants by topo III overproduction at very high levels (22).

Interestingly, one of the first mutations isolated that caused a chromosomal segregation defect mapped to a subunit of gyrase (23). The observed phenotype was named Par- and it is characterized by anucleate cells, guillotined cells and long filaments with abnormal nucleoid structures. As topo IV, not gyrase, was later shown to be the major cellular decatenase *in vivo* (24, 25), this result suggested that the gyrase mutation somehow reduced the efficiency of decatenation by topo IV. This has been explained based on the observation that negative supercoiling strongly favours the decatenation reaction of topo IV over its catenation reaction (26-29). Moreover, defective gyrase would favour the accumulation of precatenanes. Such precatenanes are good substrates for topo IV only when their density is low so that their crossing angle is optimal for enzyme activity (16). Thus, defective gyrase (and supercoiling) could lead to chromosome segregation defects by rendering topo IV inefficient for two reasons: by promoting the catenation reaction and by causing the accumulation of precatenanes.

Despite biochemical and genetic evidence for the involvement of topo III but not topo I in chromosome segregation, a defect in this process could be observed *in vivo* only when both type 1A topos were absent (30). Since only *topA* mutants, not *topB* ones, display severe growth defects it is possible that the *topA topB* phenotype reflects an absolute requirement for topo III in chromosome segregation when *topA* is absent. To test this hypothesis, we have initiated a study to understand how the various compensatory mechanisms for the absence of *topA*, such as gyrase mutations, RNase HI overproduction and others to be presented elsewhere (Usongo *et al*., manuscript in preparation), can modulate this *topA topB* phenotype. While we were testing the effect of modulating gyrase activity by using a *gyrB*(Ts) allele that is known to compensate for the absence of *topA* at

37ºC (31), we found that deleting *topA* was able to compensate for the strong gyrase inhibition at 39-40ºC and above (up to 42ºC). This temperature also coincided with the appearance of the strong Par- phenotype. This complementation by deleting *topA* was found to be totally dependent on the presence of an active *topB* gene. Here we present these results and others allowing us to conclude that topo III can play an important role in chromosome segregation *in vivo*, and that *topA* likely influences this process indirectly by regulating replication via supercoiling.

## Material and Methods

***E. coli* strains.** *S*trains used in this work are described in Table 1. Strains carrying the *gyrB*(Ts) allele carry in fact a *gyrB* gene with two mutations, one conferring coumermycin resistance (*gyrB221*) and the other one (*gyrB203*) conferring temperature sensitivity. These two mutations were obtained simultaneously following mutagenesis of a wild-type strain with N-methyl-N'-nitro-N-nitrosoguanidine (NTG). They have always been used together in our studies (4, 8, 9, 31, 33, 35, 41) and in studies from other groups (38), and DNA supercoiling is fully restored to a wild-type level when strains carrying these mutations are exposed to permissive temperatures (30°C and below; ref. 31). Strains were constructed by transduction with phage P1*vir* as previously described (32). When needed, tetracycline (10 μg/ml), or kanamycin (50 μg/ml) was added to the medium. The *gyrB*$^+$ allele was transduced into *gyrB*(Ts) recipients by selecting first for a nearby Tn10 marker and then for thermo-resistant growth (42°C). The presence of the wild-type *gyrB* allele was confirmed by sequencing.

**Plasmids.** pPH1243 is a pTrc99a derivative carrying the *topB* gene under the control of the IPTG-inducible P*trc* promoter (33). pET11-*parEC* produces a ParEC fusion protein that is active as a topo IV (34).

**Plasmid extraction for supercoiling analysis**. pPH1243 DNA extraction for supercoiling analysis was performed as described previously (35). Chloroquine gel electrophoresis and in situ hybridization of the dried gels were done as previously reported (35).

**Western blot analysis**. Western blot analysis was performed as described previously (35).

**Microscopy.** Cells were grown overnight on LB plates supplemented, when required, with cysteine (50 μg/ml) and appropriate antibiotics. When needed, IPTG (1 mM) was added to the plates. The plates were incubated at 37°C. After overnight growth, cells were resuspended in pre-warmed (37°C) liquid LB medium (supplemented as requested) to

obtain a starting $OD_{600}$ of about 0.01. Cells were grown at 37°C to an $OD_{600}$ of 0.8. The cells were prepared for microscopy as described before (35). Pictures were randomly taken and randomly selected to calculate the number of cells in each category.

**Flow cytometry**. Overnight cultures were prepared, diluted and cells were grown as indicated in either LB or M9 glucose medium supplemented as required. When the cell density reached an $OD_{600}$ of 0.3, the cells were either recovered (Fig. 1) or treated with rifampicin (300 μg/ml) to prevent the initiation of new rounds of replication and then incubated for an additional two hours, to allow the ongoing replication rounds to terminate (run-out experiments, Fig. 8). Cells were washed two times with TE buffer (Tris 10 mM, pH 8.0, EDTA 1 mM) before being fixed with ice-cold ethanol (77%). After one wash with TE buffer the cells were stained with SYTO 16 (Molecular Probes). RNase A (200 μg/ml) was also added during the staining (30 min). Flow cytometry was performed on a Becton Dickinson FACScalibur. DNA/mass ratios were obtained by dividing the average SYTO 16 green fluorescence by the average forward light scatter (FSC) that is roughly equivalent to cell mass.

# Results

**Deleting *topA* partially corrects the growth defect and the Par- phenotypes of the *gyrB*(Ts) mutant at nonpermissive temperatures.**

We found that deleting *topA* (strain RFM475) allowed a *gyrB*(Ts) mutant (strain RFM445) to grow at 42ºC on LB plates (Fig. 1a). The growth inhibition of a strain carrying the same *gyrB*(Ts) allele was previously shown to correlate with the inhibition of replication initiation at nonpermissive temperatures (36-37). Moreover, a strong Par- phenotype has been described for this allele at a nonpermissive temperature (38). Therefore, this result suggested that the absence of topo I sufficiently increased the negative supercoiling level in the *gyrB*(Ts) mutant to allow replication initiation and chromosome segregation to take place in strain RFM475.

We used flow cytometry to measure cell mass and DNA content in strains RFM445, RFM475 and RFM443 as a wild-type control. Fig. 1b shows that the average cell mass at 37ºC was similar for the three strains with that of RFM475 being slightly lower. DNA content had a somewhat wider distribution for RFM445 as compared to RFM443 and that of RMF475 was significantly wider, with the average DNA content being higher in this strain. As a result strain RFM475 clearly had a higher DNA/mass ratio (Fig. 1e, 37ºC). This could be due, at least in part, to unregulated replication in strain RFM475 (see below).

At 40 and 42ºC, strain RFM445 did not form visible colonies on plates, but gave sufficient growth in liquid (five generations) to obtain cells for flow cytometry analysis. Fig 1c shows a bimodal distribution of the average cell mass for strain RFM445 at 40ºC. This also coincided with the appearance of a high peak on the left side of the DNA histogram which corresponds to non-specific binding of the dye. This indicates the accumulation of small anucleate cells (55% of the cells were anucleate), a typical manifestation of the Par- phenotype. Moreover, DNA labeling shifted toward the right side of the histogram for strain RFM445 reflecting the presence of longer cells, another manifestation of the Par- phenotype. Based on these criteria, it can be deduced that deleting *topA* (strain RFM475) significantly corrected the Par- phenotype of the *gyrB*(Ts) strain (Fig. 1c; 9.4% of anucleate cells). This was later confirmed by fluorescence microscopy.

Fig. 1d shows that the Par- phenotype was exacerbated in both RFM445 and RFM475 at 42ºC (79.4 and 32.8% anucleate cells, respectively) as compared to 40ºC, but that deleting *topA* (strain RFM475) still significantly corrected this phenotype. In fact, the Par- phenotype was even stronger for strain RFM445 cultivated at 40ºC as compared to strain RFM475 grown at 42ºC (55.06% vs 32.8% anucleate cells, respectively). The DNA/mass ratio clearly dropped for strains RFM445 and RFM475 at 40ºC compared to 37ºC (Fig. 1e). This probably indicates reduced replication initiation due to the lack of negative supercoiling at *oriC* (14). We also noted that the drop at 42ºC was reproducibly more important for strain RFM445 as compared to RFM475 (Fig. 1e). We believe that this indicates a partial correction of the replication initiation defect in strain RFM475 conferred by the absence of *topA*. Thus, deleting *topA* significantly corrected the Par- phenotype of the *gyrB*(Ts) strain at nonpermissive temperatures plus all other supercoiling-dependent processes, e.g. replication initiation, that are required for growth.

**The correction of the growth and the Par- phenotypes of the *gyrB*(Ts) mutant by deleting *topA* depends upon the presence of the *topB* gene.**

As gyrase supercoiling activity was strongly inhibited at 40ºC and above in strains carrying the *gyrB*(Ts) allele, it was possible that topo IV could not efficiently perform DNA decatenation. In this situation, topo III activity could be required if it can actually play a role in chromosome segregation *in vivo*. The correction of the growth defect of the *gyrB*(Ts*)* strain by deleting *topA* gave us the opportunity to test this possibility. We used P1*vir* transduction to introduce a *topB* null allele into the *topA gyrB*(Ts) strain, RFM475. As a control for transduction efficiency, we also introduced the *topB* null allele within isogenic wild-type (RM443) and *gyrB*(Ts) (RFM445) strains. For these strains, *topB* null transductants were obtained after 18 hours of incubation at 37ºC, whereas 48 hours were required to obtain transductants of strain RFM475 at the same temperature. The disruption of the *topB* gene was confirmed by PCR for 8 transductants of each group (not shown).

Upon restreaking on LB plates, transductants of strains RFM443 and RFM445 formed medium-sized colonies after 18 hrs of incubation, whereas it took 48 hours for RFM475 transductants to form colonies of similar size (not shown). The vast majority of

the colonies obtained after 48 hours were homogeneous in size. We obtained similar results for strain DM800, a widely used *topA* null mutant that also contains the naturally acquired *gyrB225* compensatory mutation (6; not shown). Similar results in terms of the number of transductants and their growth rate were also reported by another group for strain DM750, a *topA* null mutant carrying the naturally acquired *gyrA224* compensatory mutation (39). Thus, *E. coli* cells lacking both type 1A topos are viable but grow slowly.

Next, we tested the ability of *topA gyrB*(Ts) cells lacking the *topB* gene to form colonies on LB plates at 35 and 42ºC. Fig. 2a shows that, whereas RFM475 cells grew better at 42ºC than at 35ºC, cells of strain CT170, a *topB* null transductant of strain RFM475, formed colonies at 35ºC but did not grow at 42ºC. The growth behaviour of strain RFM475 was previously reported and was shown to be due to the reactivation of gyrase activity as the temperature was decreased, so that the *gyrB*(Ts) allele could no longer compensate for the absence of topo I (31). The opposite behaviour of strain CT170 shows that *topB* is required for the growth of the *topA gyrB*(Ts) strain at higher temperatures.

The growth of the two isogenic strains (RFM475 and CT170) was also monitored in liquid medium at different temperatures and in some cases cell samples were recovered for DAPI staining, and prepared for fluorescence microscopy to examine cell morphology and DNA content. The growth rate at 35ºC was very similar for both strains, whereas upon raising the temperature to 40ºC it increased for strain RFM475 but did not significantly change for strain CT170 (Fig. 2b, left and right, respectively). The temperature of 37ºC was found to be the optimal temperature for the growth of the *topA topB* mutants (Fig. 2c; Usongo *et al*., manuscript in preparation). Major differences between RFM475 and CT170 strains were observed from 39ºC. At 40ºC, the growth of strain CT170 stopped during log phase at an $OD_{600}$ of 0.6 (Fig. 2c).

One typical phenotype of *topA topB* null mutants that was previously reported is the formation of very long filaments with unsegregated nucleoids having abnormal structures (30). Such filamentous cells were also observed for our *topA topB* null mutant (strain CT170; Fig. 3, long filaments (>14 chr./cell); Fig. S3 and S4). Importantly, the proportion of such cells dramatically increased as the incubation temperature was raised to 39ºC, with

a maximum being reached at 40ºC (Fig. 3, 7% vs. 24% respectively, for 37 and 40ºC; this roughly corresponds respectively, to 30% and 70% of the total cell mass). This dramatic increase correlated with temperatures at which the *gyrB*(Ts) Par- phenotype started to appear.

As expected because of the Par- phenotype, the proportion of anucleate cells also considerably increased at high temperatures (Fig. 3, 4% vs. 48% respectively, for 37 and 40ºC). Although the isogenic RFM475 strain (*topA gyrB* (Ts)) did not significantly form very long filaments at any temperature tested (Fig. S1 and S2; less than 1% at 40ºC, Fig. 3), it also produced a large proportion of anucleate cells at higher temperatures (Fig. 3, less than 0.1% vs. 40% respectively, for 37 and 40ºC). As predicted from the results shown in Fig. 1, anucleate cells accumulated at a higher frequency in strain RFM445 (Fig. S5; 0.1% vs. 67% respectively, for 37 and 40ºC). However, RFM445 produced considerably fewer of the very long filaments than CT170 (4% vs. 24% respectively, for RFM445 and CT170 at 40ºC). A *ΔtopB::Kan* derivative of RFM445 (*gyrB*(Ts) *topB*) behaved like RFM445 except that it produced a higher number of longer cells (not shown). Thus, in the *topA* deletion strain, the *topB* gene is required to alleviate the Par- phenotype related to defective gyrase activity. When *topB* is deleted, the Par- phenotype is mostly manifested as very long filaments with unsegregated nucleoids. This could be due to the fact that the absence of *topA* (increased negative supercoiling) confers to the cells the ability to grow for a while, whereas growth is much more restricted in the single *gyrB*(Ts) mutant. We can conclude that topo III at its wild-type level can perform DNA decatenation to allow chromosome segregation to take place when gyrase is defective.

**Overproducing topo IV cannot bypass the requirement for topo III activity for the growth of the *topA gyrB*(Ts) strain.**

Our data indicated that topo IV could not fully support chromosome segregation in the *gyrB*(Ts) mutant at nonpermissive temperatures, despite the increase in negative supercoiling conferred by deleting *topA*. This could indicate either that there was not enough topo IV or that it could not substitute for topo III. To address this issue, we first introduced the plasmid pET11-*parEC* (34) into strains RFM475 (*topA gyrB* (Ts)) and

CT170 (*topA topB gyrB* (Ts)). This plasmid produces a ParEC fusion protein that is fully active as a Topo IV protein *in vitro* and that was shown to complement the thermosensitive growth of *parE*(Ts) and *parC*(Ts) strains (34). We found that this plasmid was able to stimulate the growth of strain RFM475 (Fig. 4a, RFM475). This result is in agreement with the previous finding showing that topo IV overproduction can compensate for the absence of *topA* (40), and also confirmed that an active topo IV could be produced from this plasmid. The results of western blot experiments showed that topo IV as a fusion protein was indeed produced in both RFM475 and CT170 strains carrying pET11-*parEC* (Fig. 4b).

We found that overproducing topo IV did not improve the growth of strain CT170. There was even a slight but reproducible negative effect (Fig. 4a, CT170; one log drop in the efficiency of plating for cells carrying pET11-*parEC* that also had a slightly higher doubling time than CT170 cells without pET11-*parEC*). The reason for this effect is currently unknown. Nevertheless, it clearly indicates that topo IV cannot substitute for topo III in chromosome segregation when gyrase supercoiling activity is significantly impaired. This result is also in line with a previous report showing that overproducing topo IV could not complement the growth defect of DM750 and DM800 *topA*$^{-}$ cells in which the *topB* gene had been inactivated (30).

**Topo III overproduction at a very high level can correct the Par- phenotype of the *gyrB*(Ts) mutant at a nonpermissive temperature.**

The fact that the *gyrB*(Ts) strain RFM445 displays a strong Par- phenotype despite the presence of the *topB* gene, may indicate that there is not enough topo III to fully decatenate chromosomal DNA to allow segregation. To test this hypothesis, we introduced the plasmid pC18pBAD33 (a kind gift from R.J. DiGate, University of the Sciences, Philadelphia) into strain RFM445 to overproduce topo III at a very high level. This plasmid has the *topB* gene placed under the control of the strong arabinose-inducible promoter $P_{BAD}$ and a strong Shine-Dalgarno box. The same amount of RFM445/pC18pBAD33 cells from a concentrated glycerol stock were streaked on three LB plates containing ampicillin and either, arabinose (0.05%), glucose (0,2%) or no sugar. The plates were incubated for three days at 42ºC. We found that growth was essentially restricted to the beginning of the

streaks, as very few isolated colonies were obtained (not shown). This was expected as negative supercoiling is not restored by overproducing topo III. Cells from the beginning of the streaks were recovered for DAPI staining and prepared for fluorescence microscopy to examine cell morphology and DNA content.

Fig. 5a shows that when topo III was not overproduced (0,2% glucose) a large number of anucleate cells accumulated. Most strikingly, lemon shaped cells with a huge mass of unsegregated DNA at the center were produced. However, very few anucleate cells and no lemon shaped cells were produced in the absence of sugar or with 0.05% arabinose (Fig. 5b and c respectively). Fig. 5e shows by western blot that a large quantity of topo III was produced only when cells were grown on plates with arabinose or no sugar.

Since the cells were recovered after three days of incubation, we were aware of the possibility that suppressor mutations leading to the loss of the Par- phenotype could have accumulated. To test this possibility, we took cells from the LB plate no sugar that was incubated for three days, and streaked them on an LB plate with glucose. After three days of incubation cells from the beginning of the streaks were recovered for DAPI staining and prepared for fluorescence microscopy. The fact that a large number of anucleate cells and lemon shaped cells accumulated indicated that the correction of the Par- phenotype was due to topo III overproduction and not to the accumulation of suppressor mutations. Moreover, in an independent experiment we found that RFM445 strain without plasmid produced similar amount of anucleate cells and lemon shaped cells whether it was grown on LB plate with no sugar or with glucose or arabinose (not shown).

In another experiment, we used the plasmid pPH1243 in which the *topB* gene with its poor Shine-Dalgarno sequence is placed under the control of the IPTG-inducible P*trc* promoter. However, the level of topo III overproduction achieved with this plasmid after the addition of IPTG was similar to the level obtained from pC18pBAD33 in the presence of glucose (Fig. 5f, compare lanes 2 and 3). This would explain our failure to observe an effect of pPH1243 on chromosome segregation in strain RFM445 at 42ºC (not shown). Thus, overproduction of topo III at a very high level can substantially correct the Par- phenotype of the *gyrB*(Ts) strain.

**Increasing gyrase activity in a *topA rnhA gyrB*(Ts) mutant allows chromosome segregation and growth independent of topo III overproduction.**

We have previously described a *topA gyrB*(Ts) mutant in which the depletion of RNase HI (*rnhA*) activity triggered the inhibition of the supercoiling activity of gyrase and lead to chromosome segregation defects and growth inhibition (35). Similarly, this extensive supercoiling inhibition was observed in a triple *topA rnhA gyrB*(Ts) mutant (strain SB383) in which topo III overproduction from an IPTG-inducible *topB* gene on a plasmid (pPH1243), corrected both the growth and chromosome segregation defects.

To test the hypothesis that topo III acts by compensating for the weak gyrase activity in strain SB383 (*topA rnhA gyrB* (Ts)/pPH1243), we first performed P1*vir* transduction to replace the *gyrB*(Ts) allele of strain SB383 with a wild-type one. Although the *gyrB*(Ts) allele present in strain SB383 compensated for the lack of *topA*, we reasoned that the response leading to gyrase inhibition in the absence of RNase HI would be sufficient to allow a *gyrB*$^+$-derivative of SB383 to grow. Indeed, *gyrB*$^+$ transductants of strain SB383 could be obtained. Moreover, hypernegative supercoiling was not observed following the addition of a translation inhibitor (spectinomycin) to a *gyrB*$^+$ transductant (strain NF98: *topA rnhA gyrB*$^+$/pPH1243; Fig. 6a, lane 2). This treatment was previously shown to strongly stimulate hypernegative supercoiling in *topA* null mutants (41), but not when *rnhA* was absent (as in strain SB383; 35). As a control, we show that hypernegative supercoiling accumulated in strain NF88 (*topA gyrB*$^+$/pPH1243) after the addition of spectinomycin (Fig. 6a, lane 1). Steady-state supercoiling was significantly higher in strain NF98 as compared to SB383 (Fig. 6b, lane 1, SB383 and lane 2, NF98) which is in agreement with the presence of the *gyrB*$^+$ allele in strain NF98. Thus, despite the presence of the response leading to gyrase inhibition in the absence of RNase HI, gyrase supercoiling activity was higher in strain NF98 as compared to strain SB383. In a recent report, deleting the *topA* gene in an *rnhA* mutant with a wild-type gyrase was shown to generate non-viable cells (42). Presumably, the excess negative supercoiling together with the absence of RNase HI, caused the accumulation of stable R-loops that inhibited growth and precluded the expression of the cellular response leading to gyrase inhibition.

As would be predicted if increasing gyrase activity could bypass the need for topo III overproduction, strain NF98 grown in the absence of IPTG produced much less anucleate cells as compared to strain SB383 grown under the same conditions (Fig. 7; 29 and 2% of anucleate cells, respectively for strains SB383 and NF98; Fig. S6a and S7a). Cells with a low DNA content and long filaments with unsegregated nucleoids were also produced in low amounts in strain NF98. Noticeably, the suppression was so efficient in this strain, that the addition of IPTG to overproduce topo III did not further reduce the number of cells with chromosome segregation defects (Fig. 7 and S7b). As expected strain NF98, but not SB383, was able to grow overnight on LB plates in the absence of IPTG (Fig. S8a) and both strains produced similar amount of topo III protein with and without IPTG (Fig. S8b). Thus, the need to overproduce topo III for growth and chromosome segregation could be bypassed by increasing gyrase supercoiling activity in a *topA rnhA gyrB*(Ts) strain. This result further supports the interplay between gyrase and topo III in chromosome segregation.

**The absence of *topA* and *rnhA* causes unregulated replication.**

Replication initiation that takes place at *oriC* is tightly regulated so that it occurs once and only once per cell cycle (43). This process is synchronized with the "initiation mass". Unregulated replication initiation could be especially harmful when gyrase activity is suboptimal for chromosome segregation. Interestingly, flow cytometry studies have revealed asynchronous replication in the *topA* null mutant DM800 (44). We used flow cytometry in rifampicin run-out experiments with cells grown in LB medium to investigate the regulation of replication initiation in a set of isogenic strains that included RFM443 (wild-type), RFM445 (*gyrB* (Ts)) and RFM475 (*topA gyrB* (Ts)). As shown in Fig. 8a, both RFM443 and RFM445 cells contained $2^n$ chromosomes (top left and middle left panels respectively), thus showing that replication initiation was well regulated in these strains. However, the removal of the *topA* gene from the *gyrB*(Ts) strain almost completely eliminated the $2^n$ chromosomal pattern (strain RFM475; Fig. 8a, bottom left panel). Thus, the absence of topo I leads to the appearance of unregulated replication in strain RFM475.

In the absence of RNase HI, replication can initiate from R-loops at sites other than *oriC* (45). Several of these sites (*oriK*s) are located close to the *Ter* region. Replication in *rnhA* mutants was named cSDR (constitutive stable DNA replication) because it could continue for several hours following the addition of protein synthesis inhibitors. cSDR is not synchronized with the cell cycle but is sensitive to rifampicin. Our rifampicin run-out experiments of cells grown in LB medium indicated that replication in *rnhA* cells was highly unregulated, with the loss of discrete chromosomal peaks being observed (strain PH379; Fig. 8b, bottom left panel). Thus, extensive unregulated replication is expected to occur in cells lacking both *rnhA* and *topA* as is the case for strain SB383 (*topA rnhA gyrB* (Ts)/pPH1243). This might be particularly harmful for chromosomal segregation in this strain in which gyrase activity is inhibited.

If unregulated replication is problematic for the growth (and chromosome segregation) of strain SB383, culture conditions that reduce such replication should alleviate the problem. We found that strain SB383 could grow in minimal medium without the need to overproduce topo III (not shown). Under these conditions, unregulated replication was clearly reduced in both *topA* and *rnhA* null mutants. Indeed, flow cytometry in rifampicin run-out experiments revealed a near perfect $2^n$ chromosomal pattern with only one small additional peak, reflecting some asynchrony, for the *topA* null mutant (strain RFM475; Fig. 8a, bottom right panel). A near perfect $2^n$ chromosomal pattern was also obtained in run-out experiments for *rnhA* null cells grown in minimal medium (Fig. 8b, bottom right panel). Thus, our results support the hypothesis that unregulated replication due to the absence of *topA* and *rnhA* can contribute to growth and chromosomal segregation problems of cells with a defective gyrase. Therefore, the *topA* gene would indirectly affect chromosomal segregation by regulating replication.

# Discussion

**Interplay between topo III and gyrase in chromosome segregation.**

In this paper, we have presented experimental evidence for an interplay between gyrase and topo III in chromosomal segregation *in vivo*. This interplay could not be revealed until *topA* was found to correct the growth defect of the *gyrB*(Ts) mutant at nonpermissive temperatures. Growth inhibition due to defective gyrase has several causes, including inhibition of replication initiation, chromosome segregation failure (Par-), inhibition of ribosomal RNA synthesis and others (46), that all have at least in part a common denominator, namely, the lack of negative supercoiling. Thus deleting *topA* would bring negative supercoiling to a level allowing these key cellular activities to be sufficiently completed for growth to occur. However, the *topB* gene would be required due to the Par- phenotype at nonpermissive temperatures.

*topA topB* null mutants were previously shown to suffer from severe RecA-dependent chromosomal segregation defects (30). Therefore, it could be argued that the major chromosomal segregation phenotype of the *topA topB gyrB*(Ts) strain described here was the result of two independent effects: a RecA-dependent effect that was due to the absence of both type 1A topos plus a Par- related effect due to defective gyrase activity. However, for several reasons, we do not believe this to be the case. Firstly, we have recently found that compensatory mechanisms (including a *recA* deletion) that significantly correct the chromosomal segregation defects of our *topA topB gyrB*(Ts) strain at lower temperatures, had no effects at higher temperatures (Usongo *et al*., manuscript in preparation). Secondly, here we have presented strong evidence for an interplay between gyrase and topo III in two other instances: overproduction of topo III at a very high level substantially corrects the Par- phenotype of the *gyrB*(Ts) strain, and substitution of the *gyrB(Ts)* allele by a $gyrB^+$ one bypasses the need for topo III overproduction for growth and chromosome segregation in our *topA rnhA gyrB*(Ts) strain. Thus, we believe our results are consistent with an interplay between gyrase and topo III in chromosomal segregation.

While this manuscript was in preparation, a paper was published that demonstrated an interplay between topo III and topo IV in chromosome segregation in *E. coli* (47).

However, no clues were provided as to when a specific topo instead of the other one would be required to allow chromosome segregation to occur. Furthermore, based on the fact that deleting *topB* only had a minor effect on chromosomal segregation in a strain carrying the same *gyrB*(Ts) allele as the one used in the present study, the authors concluded that there were no significant interactions between topo III and gyrase in segregation. However, as shown in our paper and as stated above, a significant interplay between *topB* and this *gyrB(*Ts*)* allele could only be seen when *topA* was also deleted. Therefore, while the paper of Perez-Cheeks *et al*. (47) clearly reveals an interplay between topo III and topo IV in chromosomal segregation, our paper shows an interplay between topo III and gyrase in this process.

The interplay between topo III and topo IV in chromosomal segregation can thus be explained, at least in part, in the context of chromosomal supercoiling that is regulated by the opposing enzymatic activities of gyrase and topo I. When gyrase activity is defective, the supercoiling level is low and topo IV would not be efficient in decatenation. In this context the activity of topo III would be required. This would explain why topo IV overproduction cannot substitute for topo III in chromosomal segregation at nonpermissive temperatures, in our *topA gyrB*(Ts) strain. On the other hand, an increase in negative supercoiling conferred by deleting *topA* in the *gyrB*(Ts) strain likely improves the ability of topo IV to perform decatenation, but the supercoiling level is still too low, thus explaining why topo III is also required.

Replication-induced positive supercoiling becomes particularly problematic when convergent replication forks are about to meet at the *Ter* region of the *E. coli* chromosome. Not only does a very high level of positive supercoiling accumulate, the space on the DNA template may also become too small to accommodate binding by gyrase. It has been shown *in vitro* that topo III together with RecQ and SSB can act at converging replication forks to topologically unlink them (48). In this reaction, RecQ helicase provides the single-stranded DNA substrate to which topo III can bind. SSB (single-stranded DNA-binding protein), by interacting with both proteins, mediates the functional cooperation between RecQ and topo III. However, we found that deleting *recQ* in our *topA gyrB*(Ts) strain does not affect the ability of the *topA* deletion to complement the *gyrB*(Ts) mutant at high temperatures, thus

indicating that RecQ is not required for topo III to perform its essential function in this situation (Usongo *et al*., unpublished results).

The location where topo III actually acts *in vivo* is still unknown. As ssDNA regions are expected to form only transiently *in vivo* (this would even be more problematic when gyrase is defective) and as the abundance of topo III is normally very low, it has been difficult to pinpoint the exact site of action of topo III *in vivo*. The ability of topo III to physically interact with SSB protein, as recently shown, may explain how topo III can have access to its site of action *in vivo* (48). Indeed, SSB may efficiently bring topo III to its site of action that may be situated at the replication forks where SSB also binds. Topo III would therefore be properly located to act on precatenanes as suggested (47).

**Interplay between topo I and gyrase in chromosomal segregation.**

The severe chromosomal segregation and growth phenotypes seen here when both type1A topos were absent may indicate that topo I can substitute for topo III in chromosomal segregation. The much lower efficiency of topo I in this process as compared to topo III could be compensated for by its much higher abundance than topo III. Although this is possible, an alternative and non-mutually exclusive explanation, that may reflect a real function of topo I, would be that it indirectly affects chromosome segregation through supercoiling by limiting firing from *oriC*. By doing so, topo I would control the number of replication forks travelling on the chromosome. This would facilitate linkage removal, especially when gyrase is defective, as is often the case in *topA* mutants.

In fact, in an *in vitro oriC*-based replication system, topo III was shown to support replication fork progression and to perform the final decatenation step, whereas inhibition of replication initiation from *oriC* was the only effect seen for topo I (20, 21). Moreover, a *topA* deletion has been shown to suppress the growth defect of a *dnaA46*(Ts) mutant at the nonpermissive temperature (49) and to cause replication from *oriC* to be unregulated (44), as also shown here in our *topA* null strain. Furthermore, the replication initiation defect of the *gyrB*(Ts) strain at nonpermissive temperatures was shown here to be corrected by deleting *topA*. This support the hypothesis that the lack of topo I activity promotes replication from *oriC* by causing negative supercoiling to increase in this region, thus

facilitating DNA melting. Thus, in the *topA gyrB*(Ts) strain, unregulated replication from *oriC* would make the defective gyrase unable to efficiently support chromosome segregation. The wild-type level of topo III activity would then be indispensable, but sufficient, for chromosome segregation. Alternatively, we also have to consider the possibility that topo I, through supercoiling regulation, may affect chromosome segregation indirectly by modulating gene expression.

In the *topA rnhA gyrB*(Ts) strain SB383, the absence of RNase HI further enhanced the level of unregulated replication already caused by the absence of *topA*, by allowing replication from stable R-loops (cSDR). Moreover, since one major function of topo I is to inhibit R-loop formation (5, 8), the absence of *topA* is also expected to stimulate cSDR. Together with the fact that gyrase supercoiling activity is significantly inhibited in the absence of RNase HI (35), this high level of unregulated replication could explain why topo III needed to be overproduced in strain SB383, to allow growth and chromosomal segregation. In this context, our results suggest that the previously reported effect of *rnhA* on chromosomal segregation (35) is indirect and likely related to cSDR. Thus, topo I and RNase HI may facilitate chromosomal segregation by limiting replication.

Interestingly, when the *topA rnhA gyrB*(Ts) strain was grown in a minimal medium instead of a rich one, chromosomal segregation was significantly improved and this coincided with a clear reduction in the amount of unregulated replication conferred by deleting *topA* or *rnhA*. This correlation strongly supports the link between unregulated replication and chromosomal segregation defects. Furthermore, our results of transposon mutagenesis to isolate suppressors of the growth defect of strain SB383 indicate that indeed, unregulated replication conferred by the absence of *topA* and *rnhA* significantly contributes to the chromosomal segregation defect seen in this strain (Usongo *et al*., manuscript in preparation). Thus topo I, via supercoiling regulation, can likely affect chromosomal segregation in two ways: by affecting the efficiency of decatenation by topo IV and by regulating replication initiation.

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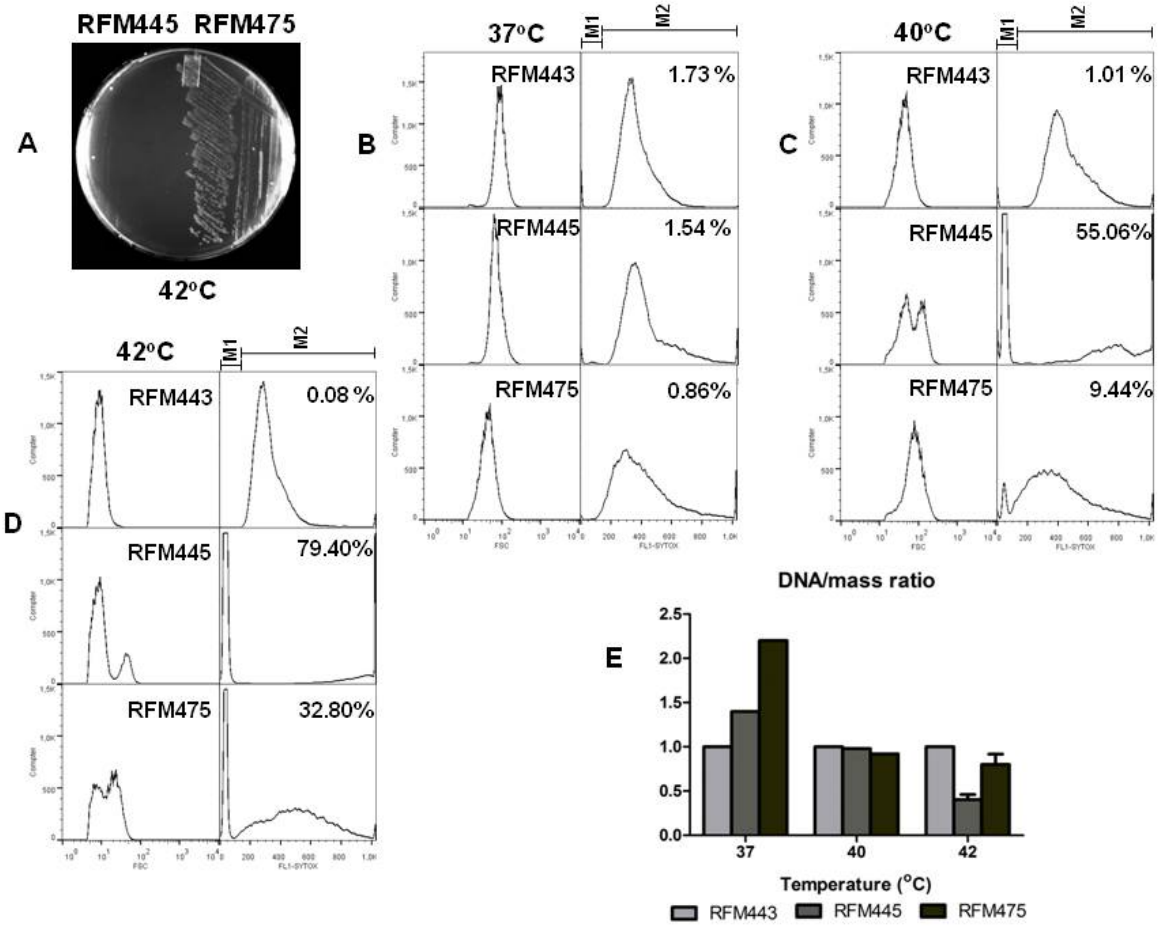
# TABLES AND FIGURES

**Table 1. *Escherichia coli* strains used in this study.**

| Strain | Genotype | Source or Ref. |
|---|---|---|
| RFM443 | *rpsL galK2 Δlac74* | 31 |
| RFM445 | *rpsL galK2 gyrB221*(cou$^R$) *gyrB203*(Ts) *Δlac74* | 31 |
| RFM475 | *rpsL galK2 gyrB221*(cou$^R$) *gyrB203*(Ts) *Δ(topA cysB)204 Δlac74* | 31 |
| NF88 | RFM475 *gyrB*$^+$ | This work |
| MD897 | DM4100 *ΔtopB::Kan* | 30 |
| CT170 | RFM475 *ΔtopB::Kan* | This work |
| RFM430 | *rpsL, galK2, ΔtrpE* | 35 |
| PH379 | RFM430 *rnhA::cam* | 35 |
| SB383 | RFM475 *rnhA::cam* pPH1243 | 35 |
| NF98 | SB383 *gyrB*$^+$ | This work |

**Fig. 1. Deleting *topA* complements the growth and Par- phenotype of the *gyrB*(Ts) strain.** a) Cells of strains RFM445 (*gyrB* (Ts)) and RFM475 (*topA gyrB* (Ts)) were grown at 37ºC to an $OD_{600}$ of 0.7 and streaked on LB plates. The plates were incubated at 42ºC for 24h. b), c) and d) flow cytometry analysis of RFM443, RFM445 and RFM475 cells grown in LB medium. Cell mass (left panels) and DNA content (right panels) parameters are shown. M1 and M2 respectively correspond to anucleate cells (the percentages are indicated in each panel) and to cells with DNA. e) DNA/mass ratios were obtained from the data shown in b) 37ºC, c) 40ºC and d) 42ºC except for strains RFM445 and RFM475 grown at 42 where the data of two additional experiments were used to obtain an average DNA/mass ratio.


RFM445 RFM475
A
42°C
37°C
M1
M2
B
RFM443
1.73 %
RFM445
1.54 %
RFM475
0.86%
40°C
C
RFM443
1.01 %
RFM445
55.06%
RFM475
9.44%
42°C
D
RFM443
0.08 %
RFM445
79.40%
RFM475
32.80%
DNA/mass ratio
E
2.5
2.0
1.5
1.0
0.5
0.0
37
40
42
Temperature (°C)
RFM443
RFM445
RFM475

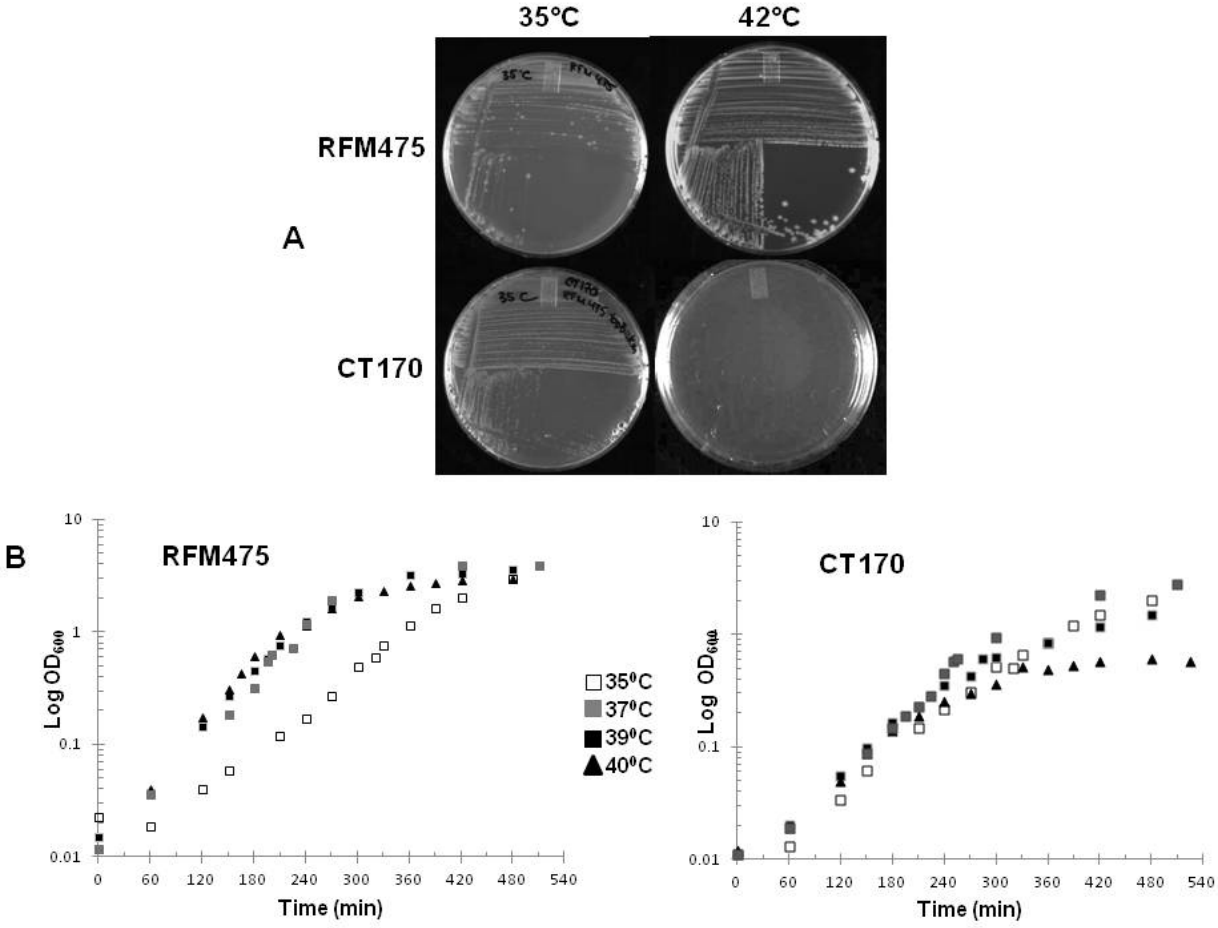



**Fig. 2. The *topB* gene is required for the growth of the *topA gyrB*(Ts) strain at nonpermissive temperatures.** a) Cells of strains RFM475 (*topA gyrB* (Ts)) and CT170 (RFM475 *topB*) were grown at 37ºC to an $OD_{600}$ of 0.7 and streaked on LB plates. The plates were incubated for 24h at 35 or 42ºC as indicated. b) Cell growth of strains RFM475 and CT170 was monitored in liquid LB at 35, 37, 39 and 40ºC as indicated.

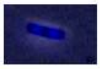
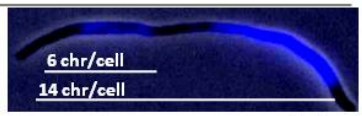
| | | Classification (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Anucleate cells | Low DNA content[1] | Small cells (1-2 chr/cell) | Short filaments (2-6 chr/cell) | Med. size filaments (6-14 chr/cell) | Long filaments (>14 chr/cell) | |
| | |  |  |  |  | | | |
| Strains | Temperature (°C) | | | | | | | Total |
| RFM475 | 37 | 0.1 | 0 | 91.9 | 7.8 | 0.2 | 0 | 832 |
| | 39 | 24.2 | 0.8 | 36.2 | 35.8 | 3.0 | 0 | 772 |
| | 40 | 40.2 | N/A | 13.8 | 38.0 | 7.0 | 1.0 | 358 |
| CT170 | 37 | 4.0 | 0.3 | 46.7 | 30.0 | 12.0 | 7.0 | 651 |
| | 39 | 56.2 | 1.3 | 1.9 | 8.4 | 18.6 | 13.4 | 521 |
| | 40 | 47.7 | N/A | 0.3 | 4.8 | 21.0 | 24.2 | 338 |

**Fig. 3. The *topB* gene is required for chromosome segregation in the *topA gyrB*(Ts) strain at nonpermissive temperatures.** Cells were grown and prepared for microscopy as described in Material and Methods. Superimposed pictures of phase contrast and fluorescence (by using Adobe Photoshop) of DAPI-stained cells from strains RFM475 (*topA gyrB* (Ts)) and CT170 (RFM475 *topB*) were used to calculate the number of cells in the different categories. Total is the number of cells that were examined to calculate the percentages of cells in each category. [1]The low DNA content category likely reflects guillotined cells, a manifestation of the Par- phenotype (23). N/A means that cells in this category were not counted.

**Fig. 4. The effect of overproducing a functional ParEC fusion protein on the growth of RFM475 and CT170 strains.** a) The effect of pET11-*parEC* on the growth of RFM475 and CT170 cells was monitored by spotting 10 µl of serial 10-fold dilutions of cells grown in LB to an $OD_{600}$ of 0.6 (at 30 and 37ºC respectively for strain RFM475 and CT170) from $10^0$ to $10^{-5}$ (from left to right) on LB plates that were incubated at 30ºC for 48h (RFM475) or 37ºC for 24h (CT170). Cells grown in liquid were also used to calculate the efficiency of plating (EOP; number of viable cells (colonies) without plasmid divided by the number of viable cells carrying pET11-*parEC*) and the doubling time. The results shown here are representative of three independent experiments. b) CT170 (lanes 1 and 2) and RFM475 cells (lanes 3 and 4) with (lanes 2 and 4) or without (lanes 1 and 3) pET11-*parEC* were grown on LB plates at 37ºC for 24 h. Aliquots of cells were recovered for Western Blots with anti-ParC (top panel) or anti-ParE (bottom panel) antibodies as described in Material and Methods.

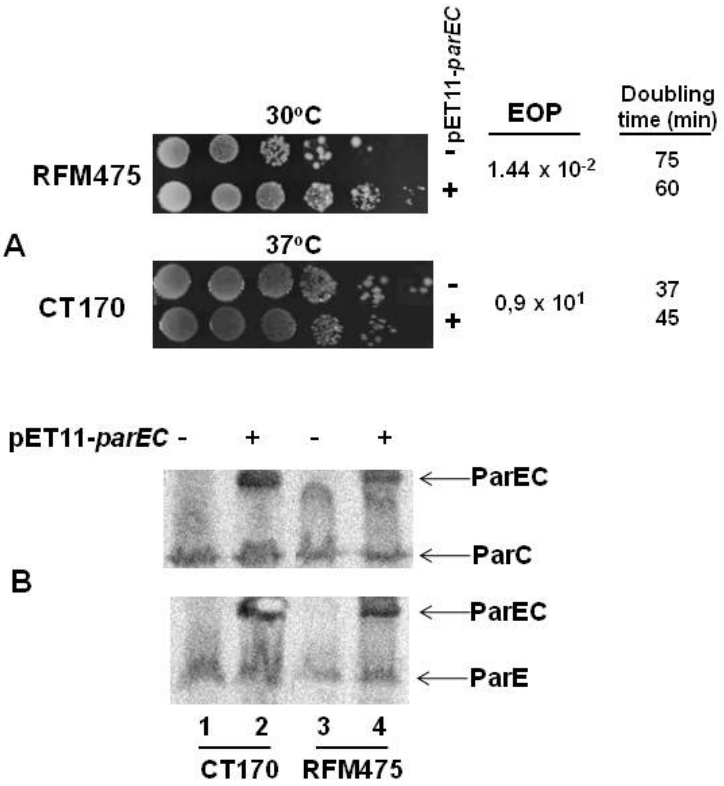
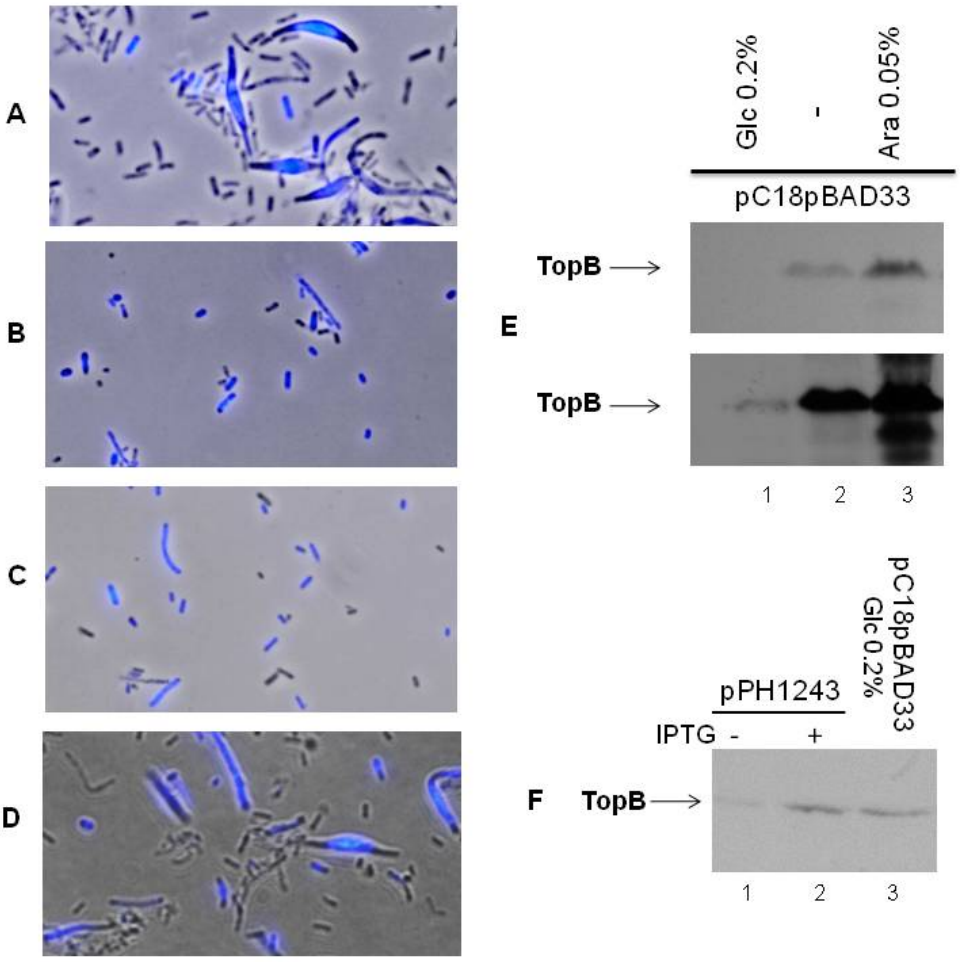

A
30°C
RFM475
37°C
CT170
pET11-parEC
-
+
-
+
EOP
1.44 x 10-2
0,9 x 101
Doubling time (min)
75
60
37
45
B
pET11-parEC - + - +
ParEC
ParC
ParEC
ParE
1 2 3 4
CT170 RFM475

**Fig. 5. Overproducing topo III at a very high level corrects the Par- phenotype of a *gyrB*(Ts) strain at 42ºC**. RFM445 strain (*gyrB* (Ts)) carrying pC18pBAD33 was grown on LB plates with glucose 0.2 % (a), no sugar added (b) or arabinose 0.05% (c) at 42ºC for 72 hours. In all cases, growth was essentially restricted to the beginning of the streaks as expected since the chromosomal DNA is extensively relaxed. Cells that were able to grow were shown not to be revertants as they kept their thermosensitive growth phenotype. Aliquots of cells were recovered for fluorescence microscopy to examine cell morphology and DNA content. Superimposed (by using Adobe Photoshop) pictures of phase contrast and fluorescence are shown. In (d), an aliquot of cells from the LB plate with no sugar was streaked on an LB plate with glucose and incubated for 72 hours at 42ºC. Growth was restricted to the beginning of the streaks. Cells were recovered for fluorescence microscopy as described above. Data shown in a) to d) are representative of three independent experiments. Western blots showing the level of topo III overproduction from cells carrying either pC18pBAD33 (e) or pPH1243 (f). In e), the bottom panel is an over-exposition of the gel to reveal the topo III band in the 0.2% glucose sample.


A
B
C
D
E
Glc 0.2%
-
Ara 0.05%
pC18pBAD33
TopB
TopB
1
2
3
pC18pBAD33
Glc 0.2%
pPH1243
IPTG -
+
F
TopB
1
2
3

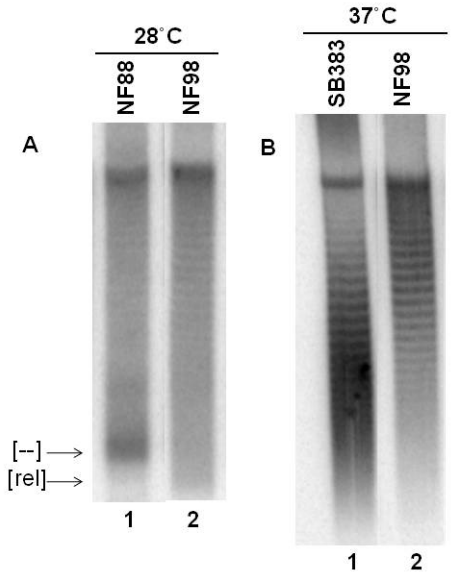



**Fig. 6. Effects on DNA supercoiling of substituting the *gyrB*(Ts) allele of strain SB383 (*topA rnhA gyrB* (Ts)) for a *gyrB*$^+$ one**. a) NF88 (RFM475 *gyrB*$^+$) and NF98 (SB383 *gyrB*$^+$) cells were grown in LB with IPTG (1 mM) at 37ºC to an $OD_{600}$ of 0.6 at which time spectinomycin (250 μg/ml) was added and 15 minutes later the cells were transferred to 28ºC for 30 minutes. pPH1243 DNA was extracted and the topoisomers were resolved following electrophoresis in an agarose gel containing 7.5 μg/ml of chloroquine. At this chloroquine concentration the relaxed topoisomers migrate more rapidly than the negatively supercoiled ones, except the hypernegatively supercoiled topoisomers that also migrate rapidly. b) SB383 and NF98 cells were grown in LB at 37ºC to an $OD_{600}$ of 0.6 at which time pPH1243 DNA was extracted and the topoisomers were resolved as described above. The gels were hybridized with a probe to detect pPH1243 topoisomers. [--] and [rel] respectively point to hypernegatively supercoiled and extensively relaxed DNA.

| | | Classification (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Anucleate cells | Low DNA content[1] | Segregated nucleoids | Short filaments[2] | Unsegregated nucleoids, long filaments | |
| | |  |  |  |  |  | |
| Strains | IPTG (1mM) | | | | | | Total |
| SB383 | + | 15.5 | 7.8 | 18.7 | 52.7 | 5.4 | 1494 |
| | - | 29.4 | 21.3 | 1.4 | 25.4 | 22.6 | 1307 |
| NF98 | + | 4.0 | 5.4 | 18.1 | 67.5 | 5.0 | 846 |
| | - | 2.3 | 6.7 | 19.6 | 67.2 | 4.2 | 741 |

**Fig. 7. Substituting the *gyrB*(Ts) allele of strain SB383 (*topA rnhA gyrB* (Ts)) for a *gyrB*$^+$ one substantially corrects the chromosomal segregation defect.** Cells were grown and prepared for microscopy as described in Material and Methods. Superimposed pictures of phase contrast and fluorescence (by using Adobe Photoshop) of DAPI-stained cells from strain SB383 (*topA rnhA gyrB* (Ts)/pPH1243) and its *gyrB*$^+$-derivative (NF98) were used to calculate the number of cells in the different categories. Total is the number of cells that were examined to calculate the percentages of cells in each category. [1]The low DNA content category likely reflects guillotined cells, a manifestation of the Par- phenotype (23). [2]Cells in the short filaments category have nucleoids that are not fully segregated. These cells were most likely viable since they increased in proportion in strains that grew better (e.g. SB383 + IPTG).

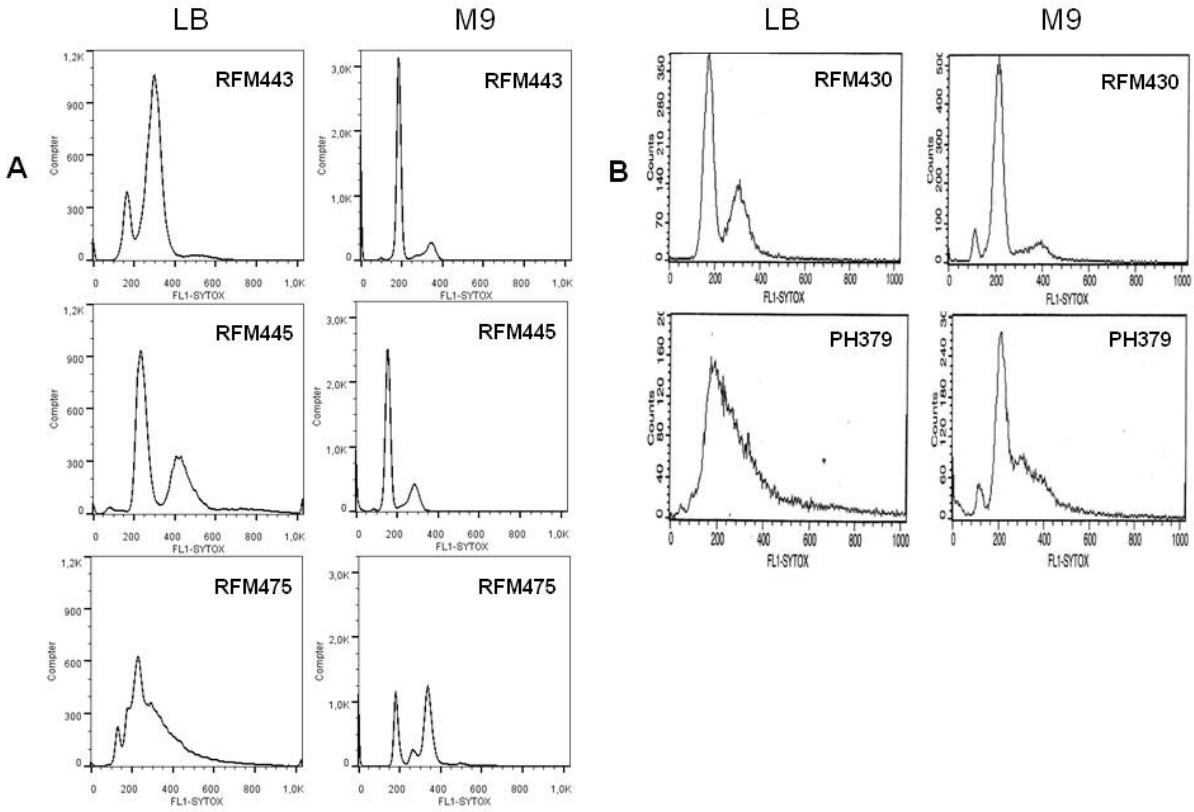



**Fig. 8. Effects of the growth medium on unregulated replication in *topA* and *rnhA* mutants.** Rifampicin run-out experiments for flow cytometry analyses were performed as described in Material and Methods. a) RFM443 (wild-type, top panels), RFM445 (*gyrB* (Ts), middle panels) and RFM475 (*gyrB*(Ts) *topA*, bottom panels) cells grown in LB (left panels) or M9 glucose (right panels) medium and b) RFM430 (wild-type, top panels) and PH379 (*rnhA*, bottom panels) cells grown in LB (left panels) or M9 glucose (right panels) medium.

# Preface to Chapter 4

In continuation of the work reported in chapter 2, we found that suppressors that restored supercoiling and corrected the growth and segregation defects of *topA rnhA gyrB*(Ts) mutants mostly mapped to genes involved in replication. This suggested that the chromosome segregation defects observed in these mutants could be linked to unregulated replication. We have already provided experimental evidence for interplay between type IA topoisomerases and gyrase in chromosome segregation. Here we wanted to find out whether type IA topoisomerases affect chromosome segregation by regulating replication. We use genetic approaches coupled with suppressor screens, spot assays and microscopy to analyse cell morphology and nucleotide shape. We found that deleting *recQ*, *recA*, and overproducing topoisomerase III corrected the growth and segregation defects of *topA* mutants. We also found that genetic suppressors of *topA rnhA gyrB*(Ts) that affected replication corrected the growth and segregation defects of *topA topB* mutants. Our data strongly suggest that bacterial type IA topos maintain the stability of the genome by preventing overreplication and by acting with RecQ to prevent RecA-dependent chromosome segregation defects.

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Author's contribution. The project was designed by Dr Marc Drolet. My contribution towards the preparation of this manuscript was significant. I performed all the experiments and equally prepared all the figures in the manuscript. I proofread the final draft prior to submission.

# CHAPTER 4: Manuscript III

**Roles of type IA topoisomerases in genome maintenance**

Valentine Usongo and Marc Drolet

Running title: Roles of type IA topoisomerases in genome maintenance

Key words: type 1A topoisomerases, topoisomerase I, topoisomerase III, chromosome segregation, supercoiling, R-loops, RecQ

# Abstract

In eukaryotes, type 1A topoisomerases (topos) act with RecQ-like helicases to maintain the stability of the genome. Despite having been the first type 1A enzymes to be discovered, much less is known about the involvement of the *E. coli* topo I (*topA*) and III (*topB*) enzymes in genome maintenance. For example, whether or not they act with RecQ is still unclear. They are thought to have distinct functions in the cell: topo I regulates supercoiling and R-loop formation, and topo III is involved in chromosome segregation. To better characterize the roles of these enzymes, we have used genetic approaches including suppressor screens, combined with microscopy for the examination of cell morphology and nucleoid shape. We show that *topA* mutants can suffer from growth inhibitory and supercoiling-dependent chromosome segregation defects. Topo III overproduction and *recA* or *recQ* deletions correct these problems. Deleting *recJ*, *recO* or *recR* acting in the RecFOR recombination pathway has no effects, nor does the overproduction of RNase HI. However, an *oriC*::*aph* suppressor mutation still *oriC*-competent in *topA* null but not in isogenic *topA*$^{+}$ cells significantly alleviates these problems. When both topo I and III are missing, excess supercoiling triggers growth inhibition that correlates with the formation of extremely long filaments fully packed with unsegregated and diffuse DNA. These phenotypes are corrected by overproducing RNase HI, by deleting *recA* but not *recQ*, or by genetic suppressors of double *topA rnhA* mutants that affect replication in general (*holC::aph*) or constitutive stable DNA replication (*dnaT*::*aph* and *rne*::*aph*), which initiates from R-loops. Altogether, our data strongly suggest that bacterial type 1A topos maintain the stability of the genome (i) by preventing the deleterious consequences of over-replication from *oriC* and R-loops and (ii) by acting with RecQ. Furthermore, topo I appear to be the primary type 1A enzyme involved in both functions.

## Author Summary

DNA topoisomerases are ubiquitous enzymes that solve the topological problems associated with replication, transcription and recombination. Eukaryotic enzymes of the type 1A family work with RecQ-like helicases such as BLM and Sgs1 and are involved in genome maintenance. Interestingly, *E. coli* topo I, a type 1A enzyme and the first topoisomerase to be discovered, appears to have distinct cellular functions that are related to supercoiling regulation and to the inhibition of R-loop formation. Here we present data strongly suggesting that these cellular functions are required to inhibit inappropriate replication originating from either *oriC*, the normal origin of replication, or R-loops that can otherwise lead to severe chromosome segregation defects. Avoiding such inappropriate replication appears to be a key cellular function for genome maintenance, since the other *E. coli* type 1A topo, topo III, is also involved. Furthermore, our data suggest that bacterial type 1A topos, like their eukaryotic counterparts, can also act with RecQ in genome maintenance. Altogether, our data provide new insight into the role of type 1A topos in genome maintenance and reveal an interplay between these enzymes and R-loops, structures that can also dramatically affect the stability of the genome as recently shown in numerous studies.

# Introduction

Type 1A topoisomerases (topos) are essential and ubiquitous enzymes found in bacteria, archaea and eukarya [1,2]. They all require single-stranded DNA (ssDNA) regions for activity. Such substrates can already be present, for example, on negatively supercoiled DNA, at the replication fork and on R-loops, or can be generated by the action of proteins, such as helicases or RNA polymerases. *E. coli* topo I, the first topo to be discovered [3], is the prototype enzyme of this family. This enzyme binds to ssDNA close to dsDNA ones [4] and is therefore well suited for the removal of excess negative supercoiling that accumulate in the wake of moving RNA polymerases [5], or following the action of DNA gyrase, the enzyme that introduces negative supercoiling within the DNA [6].

The best evidence for a major role of topo I in the regulation of supercoiling came from the observation that *topA* null mutants accumulate compensatory mutations in *gyrA* or *gyrB* genes allowing them to grow [7]. These mutations decrease the supercoiling activity of gyrase which leads to a reduction in the global chromosome supercoiling level below that of wild-type cells [8]. The role of topo I in transcription is supported by the finding that it physically interacts with RNA polymerase [9]. One major consequence of excess negative supercoiling is R-loop formation [10]. This is supported by the observation that the growth defect of *topA* null mutant can be partially compensated by RNase HI overproduction [11]. Evidence for extensive R-loop formation in the absence of topo I has been provided both *in vitro* and *in vivo* [12-15]. It is believed that topo I prevent R-loop formation mainly by relaxing transcription-induced negative supercoiling [15]. R-loops are also hot-spots for topo I activity [13].

After a temperature downshift to reactivate gyrase in a *topA* null mutant carrying a *gyrB*(Ts) allele, RNase HI overproduction was shown to prevent a transient growth arrest that correlated with the accumulation of excess negative supercoiling (hypernegative supercoiling) and extensive RNA degradation [16]. RNase HI overproduction was found both to reduce the accumulation of excess negative supercoils and to promote their rapid removal by topo IV [16,17], the other enzyme that can relax negative supercoiling in *E. coli* [18]. Moreover, evidence for R-loops impeding transcription of ribosomal RNA genes (*rrn*

operons) in *topA* null mutants has been reported [19]. Interestingly, R-loop-dependent gene expression inhibition related to RNA polymerases blockage and RNA degradation has also been reported for yeast cells lacking topo I, a type 1B topo [20]. Thus, R-loop-mediated impairment of gene expression appears to be a major mechanism by which excess negative supercoiling inhibits growth.

*E. coli* topo I is a relatively abundant protein being in the top 25% of the most abundant proteins in *E. coli* (134 ppm) [21]. The *topA* gene is under the control of promoters recognized by different sigma factors, σ32, σS and σ70 and its expression is important for *E. coli* response to various stresses including heat and oxidative shocks [22]. RNase HI overproduction was shown to partially restore the expression of σ32-regulated genes required for the heat shock response [23].

Although studies of topo I mostly focused on its role in supercoiling regulation and its effect on gene expression, evidence for the involvement of this enzyme in other DNA transactions such as chromosome segregation and replication initiation has been provided [17,24-27]. Interestingly, one of the first function to be proposed for topo I was a role as a specificity factor to inhibit non-*oriC* replication initiation, such as initiation from R-loops, that could occur in an *in vitro* reconstituted system for *oriC*-dependent replication [28]. However, there is no experimental evidence for such a role of topo I *in vivo*.

*E. coli* topo III, the second type 1A topo to be discovered, has a much higher preference for ssDNA than *E. coli* topo I [29]. As a consequence, topo III is very inefficient in relaxing DNA with a physiological supercoiling density and, in fact, this enzyme plays no role in supercoiling regulation [18,30]. However, topo III was shown to be a very potent decatenase during replication *in vitro* provided that a ssDNA region was present on the DNA substrate for the binding of the enzyme [29]. The presence of a unique amino-acid sequence in the topo III protein named the “decatenation loop” (absent in eukaryotic type 1A enzymes), was found to be essential for the decatenation of replication intermediates [31].

Unlike topo I, topo III is a protein of low abundance (9.4 ppm) [21]. Moreover, as opposed to *topA* null mutants, cells lacking topo III activity display no growth defects [32]. Recently, it has been shown that topo III plays a role in chromosome segregation *in vivo*

that is likely related to replication, as this function was shown to be mostly required when the activity of topo IV [33], the main cellular decatenase, or gyrase [34] were severely impaired. Topo III physically interacts with SSB protein and this interaction presumably allows topo III to act at the replication fork in the cell [35].

Similar to eukaryotic type 1A topos (see below), topo III activity was shown to be stimulated by RecQ helicase *in vitro* [35-37], but these two proteins do not physically interact. Evidence for RecQ acting with topo III in *E. coli* cells has been reported [30]. However, because some important properties of the strains used in this work could not be observed in an independent study, the conclusion that RecQ acts with topo III has been questioned [33].

*Saccharomyces cerevisiae* type 1A topo was the first enzyme of this family to be discovered in eukaryotic cells [38]. Being the third topo identified in this organism, it was named Top3. The existence of this topo was revealed following the isolation of a mutation, in *top3*, that stimulated recombination between repeated sequences [38]. Interestingly, phenotypes of *top3* mutants including slow growth and sporulation deficiency were suppressed to different extents by inactivating *SGS1*, encoding the RecQ homolog of *S. cerevisiae*, or by overproducing *E. coli* topo I [38-40]. Moreover, deleting *RAD51*, encoding the RecA homolog of *S. cerevisiae*, was shown to rescue the slow growth phenotype of *top3* mutants [41]. Altogether, these data suggested that Sgs1 processed recombination intermediates to generate structures that could only be resolved by a type 1A topo, such as Top3 or *E. coli* topo I.

Physical interactions between type 1A topos (named topo III in higher eukaryotes) and their RecQ-like partner from eukaryotic organisms (e.g BLM in human and in *Drosophila*) have been demonstrated [1,39,42,43]. It is now well established that these complexes can efficiently resolve homologous recombination intermediates (Double Holliday Junctions; DHJs) without genetic exchange [1,44-46]. Reactions of BLM with topo III are often stimulated by the presence of RPA, the SSB homolog of eukaryotes that presumably stabilizes the BLM-generated ssDNA region, the substrate for topo III [44,46]. Eukaryotic topo III enzymes have a higher requirement for ssDNA than *E. coli* topo I and,

in fact, they are generally considered to be more closely related to *E. coli* topo III than topo I [1].

An interplay between *E. coli* topo I and III has been reported in two instances. In the first one, the *topB* gene was isolated as a multicopy suppressor of a *topA* null mutant [47]. In this study, *in vitro* experiments were performed in which an R-looped DNA substrate was found to be a relatively better substrate for relaxation by topo III than topo I, whereas topo I was found to be more efficient than topo III in relaxing transcription-induced negative supercoiling. In fact, *in vivo*, topo III was found to relax only poorly the excess negative supercoiling introduced by gyrase. This is consistent with our observation that topo III overproduction, unlike RNase HI overproduction, is unable to prevent the supercoiling-dependent transient growth arrest of a *topA gyrB*(Ts) strain, following a temperature downshift ([16]; Baaklini and Drolet, unpublished).

These results might suggest that topo III overproduction complemented a yet unknown function of topo I that was not directly related to excess supercoiling. Indeed, here we present genetic evidence for an important role of topo I acting with RecQ to resolve RecA-dependent recombination intermediates, that otherwise inhibit chromosome segregation. Moreover, our data suggest that the requirement for this activity is related to over-replication mostly from *oriC* that takes place in the absence of *topA*.

In the second instance, deleting *topB* from a *topA* null mutant carrying a *gyrA* or *gyrB* compensatory mutation, leads to the formation of very long filaments with unsegregated nucleoids having abnormal structures and, eventually, to growth arrest [48]. Here, our data suggest that these phenotypes are exacerbated by excess negative supercoiling and are related to R-loops and over-replication. Once again, they point to a major role for topo I in preventing inappropriate replication. In this context, topo III may also play a role in inhibiting unnecessary replication initiated outside of *oriC*. Thus, *E. coli* type 1A topos are required to prevent the deleterious consequences of unregulated replication.

# Results

**Supercoiling-dependent growth and chromosome segregation defects in cells lacking topo I activity.**

To look for chromosome segregation defects in a *topA* null mutant, cells of a *ΔtopA gyrB*(Ts) strain were stained with DAPI and prepared for microscopy such that both cell morphology and DNA content could be examined. By growing the cells at 30ºC, the permissive temperature for gyrase, we could test the true effect of losing *topA* on nucleoid shape. As can be seen in Fig. 1a, whereas nucleoids of *gyrB*(Ts) cells (RFM445) were well separated and compact, those of isogenic *topA gyrB*(Ts) cells (RFM475) were less compact and clearly not separated, thus showing chromosome segregation defects.

To verify if these problems were related to excess negative supercoiling, *topA* null cells of strain RFM475 were grown at 37ºC so that gyrase activity was reduced. At this temperature the chromosome supercoiling level decreases below that of wild-type cells and, as a result, *topA* null cells can grow robustly [11,49]. At 37ºC, chromosome segregation in RFM475 strain was significantly improved, as many cells had well separated and more compact nucleoids as compared to cells grown at 30ºC (Fig. 1a, RFM475, 37ºC vs RFM475). We tested the effect of RNase HI overproduction on chromosome segregation in strain RFM475 grown at 30ºC. It did not correct the chromosome segregation defect (RFM475/pSK760). Thus, we conclude that *topA* null cells suffer from supercoiling-dependent chromosome segregation defects that seem to be unrelated to R-loops.

RNase HI overproduction did not correct the chromosome segregation problem whereas it clearly stimulated the growth of *topA gyrB*(Ts) null cells at 30ºC (Fig. 1b, RFM475 vs RFM475/pSK760). Therefore, at this temperature the defect was not strong enough to offset the positive effect of overproducing RNase HI. We have previously shown that RNase HI overproduction could not complement the growth defect of *topA* null mutants at lower temperatures. In fact, it had a negative effect [47,50]. The cold sensitivity of *topA* null mutants was found to be, at least in part, related to the inability of topo IV to efficiently relax negative supercoiling at low temperatures [16,17]. As a result, hypernegative supercoiling accumulated.

We found that the chromosome segregation defect of our *topA gyrB*(Ts) strain was exacerbated at 24ºC since the cells were generally longer and the DNA more diffuse as compared to cells grown at 30ºC (RFM475, Fig. 2a, 24ºC vs Fig. 1a, 30ºC). Overproducing RNase HI further stimulated cell filamentation and produced cells with large DNA-free regions (Fig. 2a, RFM475/pSK760). Growth of *topA gyrB(*Ts) cells on solid LB medium at 24ºC was very poor whether RNase HI was overproduced or not (Fig. 2b). Thus, the cold sensitivity of *topA* null cells triggered by excessive hypernegative supercoiling correlates with a strong chromosome segregation defect that is exacerbated by RNase HI overproduction.

**Topo III overproduction and *recA* or *recQ* deletions correct both the growth and chromosome segregation defects in cells lacking topo I activity.**

Topo III overproduction was previously shown to partially restore the growth defect of *topA* null mutants at low temperatures [47]. Moreover, not only it counteracted the negative effect of RNase HI overproduction, increasing topo III activity allowed the positive effect of RNase HI overproduction to be revealed [47]. An additive effect on the growth of *topA* null mutants at 28 and 21ºC was observed following the simultaneous overproduction of topo III and RNase HI both in liquid and on solid media [47]. In fact, overproducing RNase HI in liquid media was shown to stimulate the growth of *topA* null mutants and to partially correct the RNA synthesis defect at 21ºC [16]. Therefore, the fact that RNase HI overproduction did not promote but rather inhibited colony formation on solid media, suggested that the growth stimulation by RNase HI eventually exacerbated the chromosome segregation defect of *topA* null cells. This would imply that topo III overproduction complemented the growth defect of *topA* null cells by correcting their chromosome segregation defects.

This turned out to be true as we found that overproducing topo III almost fully, at 30ºC, or partially, at 24ºC, corrected the chromosome segregation defect of *topA gyrB*(Ts) cells (RFM475/pPH1243; fig. 1a, at 30ºC the nucleoids are well separated and compact; fig. 2a, at 24ºC some nucleoids separated, shorter cells and DNA more compact as compared to cells not overproducing topo III). As expected, topo III overproduction also

promoted growth on solid media at these temperatures (Fig 1b and 2b). Thus, topo III overproduction corrects the growth defect of *topA* null mutants, at least in part, by facilitating chromosome segregation.

Since type 1A topos from different organisms have been shown to act with RecQ-like helicases, we tested the effect of deleting *recQ* on the growth and chromosome segregation in *topA* null cells. We found that deleting *recQ* was as good as overproducing topo III in correcting the growth defect of our *topA gyrB*(Ts) strain (RFM475) at both 30 and 24ºC (Fig. 1b and 2b; RFM475 *recQ*; Western blot experiments showed that topo IV was not overproduced in the *topA* null strain lacking *recQ*; Fig. S3). Deleting *recQ* also partially corrected the chromosome segregation defect at these temperatures (Fig. 1a and 2a). Thus, our results suggest that *recQ* and *topB* act in a pathway that is related to chromosome segregation in the absence of *topA*.

Quite often RecQ-like helicases and type 1A topos act together in the context of homologous recombination [1,51]. We therefore tested the effect of deleting *recA* on the growth and chromosome segregation in *topA* null cells. We found that deleting *recA* partially corrected the growth defect of our *topA gyrB*(Ts) strain at both 30 and 24ºC (Fig. 1b and 2b; RFM475 *recA*), though the effect was not as good as the one conferred by deleting *recQ* or overproducing topo III (RFM475 *recA* vs RFM475 *recQ* and RFM475/pPH1243). In fact, the positive effect of deleting *recA* on the growth of *topA* null cells at 24ºC was better seen after three days of incubation (not shown). Deleting *recA* also partially alleviated the chromosome segregation defects of *topA* null cells at both temperatures (Fig. 1a, 30ºC and Fig. 2a, 24ºC). These results demonstrate that the chromosome segregation defects of *topA* null mutants are largely RecA-dependent and therefore support the involvement of homologous recombination.

As shown above, overproducing topo III and RNase HI improved the growth of *topA* null mutants for two different reasons. As a result, when they were overproduced simultaneously, they had an additive effect on the growth of *topA* null cells [47]. We found that overproducing topo III had no effects on the growth of our *recQ*- or *recA*-derivatives of the *topA gyrB(*Ts) strain (Fig. 3, RFM475 *recA* and *recQ*, + or – pPH1243)). These results suggest that topo III, RecQ and RecA act in the same pathway and, furthermore, that topo

III acts after RecA and RecQ. This would be consistent with RecQ processing RecA-generated recombination intermediates in such a way that they can only be resolved by a type 1A topo. Since topo III needs to be overproduced, we believe that the much more abundant topo I enzyme is normally involved in the resolution of these intermediates (see Discussion).

In *E. coli*, positive effects of deleting *recQ* on growth and chromosome segregation are sometimes attributed to unnecessary RecA-mediated recombination via the RecFOR pathway, at arrested replication forks [52,53]. In this pathway, RecQ helicase acts with RecJ, a 5'-3' exonuclease, to provide ssDNA regions on which RecF, O and R facilitate RecA nucleoprotein filament assembly. We found that deleting *recJ*, *recO* or *recR* had no effect on growth and chromosome segregation in our *topA gyrB*(Ts) strain (Fig. 1 and Fig. 2b, RFM475 *recO*; data not shown for *recJ* and *recR*). This indicated that the RecFOR pathway was not involved and, therefore, further support the involvement of RecQ with type 1A topos in resolving RecA-generated recombination intermediates.

**An *oriC::aph* mutation still *oriC* competent in *topA* null but not in isogenic *topA*+ cells complements both the growth and chromosome segregation defects**.

Our data suggested that hypernegative supercoiling in *topA* null mutants triggered RecA-dependent recombination that lead to the accumulation of RecQ-processed intermediates. Without a sufficient amount of type 1A topo activity to resolve these intermediates, chromosome segregation could not occur. However, how excess negative supercoiling stimulated RecA-dependent recombination to a level that caused chromosome segregation defects is unclear.

We have recently used a Tn*5* mutagenesis system to isolate genetic suppressors of the growth defect of a *topA rnhA gyrB*(Ts) strain (Materials and Methods; Usongo and Drolet, manuscript in preparation). The growth defect of this strain was previously shown to be related to chromosome segregation problems that could be corrected by overproducing topo III [17]. Improving gyrase activity also suppressed the chromosome segregation defects [17]. Moreover, our study of replication in this mutant lead us to speculate that unregulated replication either from *oriC* or R-loops, or from both, could

contribute to the segregation defects [34]. In agreement with this hypothesis, insertion mutants were found in loci involved in replication.

In one mutant, the *kan*$^r$ cassette was found to be inserted within the *oriC* region, close to the middle (Fig.4a, *aph*). It was possible that the suppressed strain could survive without an active *oriC* region, as replication could occur from R-loops due to the absence of the *rnhA* gene (constitutive stable DNA replication, cSDR) [54]. Therefore, to verify if this *oriC15*::*aph* mutation was still competent for replication initiation, we tried to introduce it in wild-type (RFM443), *gyrB*(Ts) (RFM445) and *topA gyrB*(Ts) (RFM475) isogenic strains. Kanamycin resistant transductants were readily obtained for the *topA* strain (RFM475). Southern blot analysis confirmed that the *topA* transductants carried the mutated (Fig. S4, RFM475 *oriC15*::*aph*) but not the wild-type *oriC* region. The few kanamycin resistant transductants of the wild-type (RFM443) and *gyrB*(Ts) strains (RFM445) were found not to be good as they kept the wild-type *oriC* region ((Fig. S4, a false positive RFM443 *kan*$^r$ is shown). We repeated the transduction experiment several times and obtained similar results. Therefore, we concluded that the *oriC15*::*aph* mutation was viable only when the *topA* gene was absent.

Our finding that overproducing RNase HI had no effect on the growth of the *topA* null strain carrying the *oriC15*::*aph* mutation (at 37 and 41$^o$C, not shown), indicated that this strain does not replicate its chromosome via cSDR. This is in agreement with a previous report showing that, as opposed to an *rnhA* null mutant, a *topA* null mutant could not survive without a functional *oriC*/DnaA system [27]. Therefore, our *topA* null mutant very likely use the *oriC15*::*aph* allele to initiate the replication of its chromosome. However, we can predict that this allele would be less active than a wild-type one and therefore should be able to complement the growth defect of a strain in which excess replication from *oriC* is growth inhibitory. The *dnaAcos* mutant, isolated as an intragenic suppressor of a *dnaA46* mutant, fails to grow at 30$^o$C and below, because of excessive replication initiation from *oriC* [55]. We found that a *dnaAcos* strain carrying the *oriC15*::*aph* mutation could grow well at both 37 and 30$^o$C whereas an isogenic strain with a wild-type *oriC* region could not (Fig. S5, *dnaAcos oriC15::aph* vs *dnaAcos*). Thus, this

result confirmed that (i) the *oriC15*::*aph* mutation is functional in replication initiation and (ii) it is less active than a wild-type *oriC* region.

Our results with the *oriC15*::*aph* mutation suggested that topo I may play an important role in regulating replication initiation from *oriC*. In a previous study, the left-half of the *oriC* region was shown to be essential for *oriC* function *in vivo* [56]. This section carries the DUE (DNA unwinding element, AT-rich) region from which *oriC* duplex melting is initiated (Fig. 4a) [57]. The smallest *oriC* fragment found to be functional *in vivo*, was a fragment encompassing nucleotide 1 to 163 of the *oriC* region (Fig. 4a, *oriC231*). However, a wild-type strain carrying this fragment was sensitive to rich media (LB). It was concluded that the right-half of *oriC* was essential for multi-forked replication that is required to support high growth rates in rich media [56]. Therefore, the fact that the *kan*$^{r}$ cassette was inserted at position 142 in the *oriC* sequence (Fig. 4a), likely explains why our *oriC15*::*aph* mutation was not functional in a wild-type strain. However, not only the mutation was *oriC*-competent in our *topA* null mutant, it apparently allowed multi-forked replication, since our strain was able to grow robustly in rich media. Therefore, these results suggest that topo I plays an important regulatory role at *oriC*.

We used flow cytometry in rifampicin run-out experiments with cells grown in M9 medium at 37$^{o}$C to investigate the regulation of replication initiation in our strains. As recently shown [34], both wild-type (RFM443) and *gyrB*(Ts) (RFM445) cells contained 2$^{n}$ chromosome, thus indicating that replication initiation was well regulated in these strains (Fig. 4b). Cells of the *topA gyrB*(Ts) strain (RFM475) had a near perfect 2$^{n}$ chromosomal pattern with one small additional peak, showing some asynchrony (Fig. 4b). However, flow cytometry analysis revealed that replication initiation was not well regulated in the *topA* null mutant carrying the *oriC15*::*aph* mutation, as peaks reflecting 1, 2, 3, or 4 chromosomes were clearly observed (Fig. 4b, RFM475 *oriC15*::*aph*). Highly asynchronous replication was also previously detected in a wild-type strain carrying the *oriC231* mutation [56]. Flow cytometry analysis also revealed that the DNA/mass ratio was higher by roughly 40% in the *topA gyrB*(Ts) strain (RFM475) as compared to wild-type (RFM443) and *gyrB(Ts)* (RFM445) strains (Fig. 4c). Introducing the *oriC15*::*aph* mutation within the *topA* null strain restored the DNA/mass ratio to the level seen in RFM443 and RFM445

(Fig. 4c, 475 *oriC*). Thus, the *oriC15*::*aph* mutation caused replication initiation to be less efficient in the *topA* null strain as shown by the loss of regulation and the lower DNA/mass ratio.

We found that the *oriC15*::*aph* mutation very well corrected the growth defect of our *topA gyrB*(Ts) strain (RFM475) at both 30 and 24ºC (Fig 1b and 2b, respectively, RFM475 *oriC*). This mutation also significantly corrected the chromosome segregation defects of the *topA* null strain at both temperatures (Fig 1a and 2a, respectively, RFM475 *oriC*). Therefore, the *recA*-dependent chromosome segregation defects in the *topA* null mutant is likely related to excess replication from *oriC*. We conclude that one major role of *E. coli* topo I in genome maintenance is to prevent over-replication originating from *oriC*.

**Supercoiling- and R-loop-dependent growth and chromosome segregation defects in a *topA topB gyrB*(Ts) strain.**

We have recently shown that deleting *topA* could complement the growth defect of our *gyrB*(Ts) strain at nonpermissive temperatures (40 to 42ºC), by partially correcting its replication initiation and chromosome segregation defects [34]. However, we found that the *topB* gene was required for chromosome segregation and overproducing topo IV, the main cellular decatenase, could not substitute for *topB*. These results, and others, allowed us to conclude that topo III plays a role in replication that becomes essential when gyrase activity is defective. Here, we have confirmed that recombination was not involved by showing that deleting *recA* or *recQ* did not correct the growth and chromosome segregation defects of the *topA topB gyrB*(Ts) strain at a nonpermissive temperatures (40ºC, Fig. S6). Moreover, RNase HI overproduction had no effects. Thus, at nonpermissive temperatures for the *gyrB*(Ts) allele, the growth and chromosome segregation defects of the *topA topB gyrB*(Ts) strain [34] are unrelated to recombination and R-loops.

We noticed that the optimal temperature for the growth of the *topA topB gyrB*(Ts) strain was 37ºC. Indeed, at 30ºC the growth defect was found to be exacerbated (Fig. S7a, RFM475 *topB*/pSK762c vs RFM475 *topB*/pSK760, at 37 and 30ºC). We also found that this strain generated a higher proportion of longer cells at 30 than 37ºC (Fig. S7b, RFM475 *topB*, 37 vs 30ºC). Since gyrase was re-activated at 30ºC, we considered the possibility that

deleting *topB* exacerbated *topA* phenotypes at this temperature. If this was true, overproducing RNase HI should have a positive effect on growth and chromosome segregation in our triple mutant. Indeed, this turned out to be true as the spot assay revealed that growth was better, by at least two logs, when RNase HI was overproduced (Fig. 5b, RFM475 *topB*/pSK760 vs RFM475 *topB*/pSK762c). Moreover, the strong chromosome segregation defects illustrated by the formation of very long filaments fully packed with diffuse DNA, were significantly corrected by overproducing RNase H. In this case, cells were shorter and the DNA was more compact (Fig. 5a). Thus, R-loops-related problems of a *topA* null mutant were exacerbated by deleting *topB* and were mostly expressed as chromosome segregation defects.

**RecA-dependent but RecQ-independent growth and chromosome segregation defects in a *topA topB gyrB*(Ts) strain at 30°C.**

We found that deleting *recA* significantly improved the growth of the *topA topB gyrB*(Ts) strain at 30°C, though the effect was not as good as the one conferred by overproducing RNase HI (Fig. 5b, RFM475 *topB recA*/pSK762c vs RFM475 *topB*/pSK760). However, deleting *recA* was at least as good as overproducing RNase HI in correcting the chromosome segregation defects of the *topA topB gyrB*(Ts) strain (Fig. 5a, RFM475 *topB recA*/pSK762c vs RFM475 *topB*/pSK760). Furthermore, overproducing RNase HI had no effects on growth and chromosome segregation when *recA* was deleted (Fig. 5a, RFM475 *topB recA*/pSK760 vs RFM475 *topB recA*/pSK762c). These results demonstrate that the R-loop-dependent chromosome segregation defects in cells lacking type 1A topos, are also dependent on RecA.

Unlike inactivating *recA*, we found that deleting *recQ* did not correct the phenotypes of the *topA topB gyrB*(Ts) strain (Fig. 5a and b, RFM475 *topB recQ*/pSK762c vs RFM475 *topB*/pSK762c). However, RNase HI overproduction was still able to correct these phenotypes when *recQ* was absent (RFM475 *topB recQ*/pSK760 vs RFM475 *topB recQ*/pSK762c). Thus, the RecA- and R-loop-dependent growth and chromosome segregation defects of the *topA topB gyrB*(Ts) strain are not caused by the accumulation of RecQ-processed recombination intermediates that are substrate for type 1A topos. As RecA

was previously shown to be required for cSDR that initiates from R-loops [54], over-replication could possibly be the triggering event for the growth and chromosome segregation defects of cells lacking type 1A topos. This is supported by the genetic evidence presented below.

**Suppressor mutations affecting R-loop- and/or *oriC*-dependent replication significantly correct the growth and chromosome segregation defects in *topA topB gyrB(Ts)* strains at 30°C.**

One of the best suppressors of the growth defect of the *topA rnhA gyrB*(Ts) strain that has cell filamentation and chromosome segregation phenotypes similar to our *topA topB gyrB*(Ts) strain, had the *kan*$^r$ cassette inserted within the promoter region of the *dnaT* gene (Fig. S8a). DnaT is one of the various proteins that constitute the primosome (PriA-dependent [58]. This protein complex allows the assembly of a replisome outside of *oriC*. Interestingly, the first mutation found to inhibit SDR mapped within *dnaT* [59]. The SOS-dependent form of stable DNA replication (iSDR) was shown to be inhibited in this case [54]. However, the involvement of *dnaT* in the R-loop-dependent form of SDR (cSDR) is still unknown [60]. To test this, we introduced the *dnaT18*::*aph* mutation in a *dnaA46*(Ts) strain also carrying an *rnhA* null mutation. The absence of RNase HI allows the *dnaA46*(Ts) strain to grow at 42°C as it can replicate its chromosome from R-loops (Fig. 6 a and b). Therefore, the fact that the *dnaT18*::*aph* allele inhibited the growth of the *dnaA46*(Ts) *rnhA* strain at 42°C (Fig. 6c, 42°C, *rnhA dnaA46* vs *rnhA dnaA46 dnaT*) indicated that the *dnaT* gene was required for cSDR.

The *dnaT18*::*aph* mutation was also found to partially correct the chromosome segregation defects of the *topA rnhA gyrB*(Ts) strain (Fig. S9). This indicated that replication from R-loops could, at least in part, be responsible for these problems. We therefore tested the ability of the *dnaT18*::*aph* mutation to correct similar defects in cells lacking type 1A topos. For this purpose, we used a different null allele of *topA*, the *topA20*::Tn10 allele that was previously shown to behave similarly to the *ΔtopA* allele used in the present study [11]. We first constructed a *topB gyrB*(Ts) strain in which the *topA20*::Tn10 allele was either immediately introduced to obtain the *topA topB gyrB*(Ts)

control strain (VU421), or introduced after the *dnaT18*::*aph* allele to obtain the *topA topB gyrB*(Ts) *dnaT18*::*aph* strain (VU441). We found that the chromosome segregation defects were more severe in our new *topA topB* strain (VU421) as compared to the other one carrying the *ΔtopA* allele (Fig. 5a, RFM475 topB/pSK762c vs Fig. 7a, RFM445 *topB topA* and data not shown). Indeed, strain VU421 at 30ºC produced almost exclusively extremely long filaments that were fully packed with diffuse DNA. This could be related to our previous observation that R-loops-related problems in the absence of topo I were more severe in strains carrying the *topA20*::Tn10 allele instead of the *ΔtopA* one [61]. RNase HI overproduction also significantly corrected both the growth and chromosome segregation defects of strain VU421 (Fig. 7a and b, RFM445 *topB topA* vs RFM445 *topB topA*/pSK760). However, at 24ºC, RNase HI overproduction had no effects (Fig. 7c, RFM445 *topB topA*/pSK760 vs RFM445 *topB topA*/pSK762c). This was expected, as the cold-sensitivity of cells lacking topo I is not corrected by RNase HI overproduction (see above).

We found that the *dnaT18*::*aph* mutation was at least as good as RNase HI overproduction in correcting the chromosome segregation defects of strain VU421 (Fig. 7a, RFM445 *topB dnaT topA* vs RFM445 *topB topA*/pSK762c and RFM445 *topB topA*/pSK760). However, RNase HI overproduction was slightly better than the *dnaT18*::*aph* mutation to correct the growth defect of strain VU421 (Fig. 7b, RFM445 *topB topA*/pSK760 vs RFM445 *topB dnaT topA*). We also found that *dnaT18*::*aph* had a negative effect on the growth of strain VU421 at 37ºC (Fig. 7d, RFM445 *topB dnaT topA* vs RFM445 *topB topA*). This could be due to the presence of the *gyrB*(Ts) allele that was previously shown, at this semi-permissive temperature, to be incompatible with a mutation (*priA* null) inactivating the primosome [62]. Thus, our results support the hypothesis that the R-loop and RecA-dependent chromosome segregation defects in cells lacking type 1A topos are, at least in part, related to over-replication initiated from R-loops. The fact that the *dnaT18*::*aph* mutation slightly promoted the growth of our *topA* null mutant (Fig. 1b and 2b, 30 and 24ºC respectively, RFM475 *dnaT* vs RFM475), suggests that cSDR is primarily a problem for *topA* null cells that is exacerbated by deleting *topB*. This would be consistent

with the assumption that topo I is the primary type 1A topo involved in the inhibition of R-loop formation [47].

We have isolated seven different *kan*$^r$ insertion mutations in the C-terminal region of RNase E, the main endoribonuclease in *E. coli* (Valentine and Drolet, manuscript in preparation), that suppressed the growth defect of our *topA rnhA gyrB*(Ts) strain. Interestingly, experimental evidence for an interplay between RNase HI and RNase E in RNA degradation has been reported [63,64]. One of these *rne* mutations (*rne59::aph*, Fig. S8c) was introduced in a *dnaA46*(Ts) *rnhA* strain to test its effect on cSDR. We found that the presence of the *rne59::aph* mutation significantly reduced the ability of the *dnaA46*(Ts) *rnhA* strain to grow at 42ºC (by 2 to 3 logs; Fig. 6d, 42ºC, *rnhA dnaA46* vs *rnhA dnaA46 rne*). This result shows that the mutated RNase E inhibited cSDR.

We constructed a *topA topB gyrB*(Ts) strain, with the *topA20*::Tn*10* allele as described above, that carried the *rne59::aph* mutation. We found that the *rne59::aph* mutation was slightly better than RNAse HI overproduction to correct the growth defect of cells lacking type 1A topos (Fig. 7b, RFM445 *topB topA rne* vs RFM445 *topB topA*/pSK760). Furthermore, it was at least as good as RNase HI overproduction and the *dnaT18*::*aph* mutation to correct the chromosome segregation defects in these cells (Fig. 7a). Thus, our results with the *rne59::aph* mutation lend further support to the hypothesis that cells lacking type 1A topos suffer from excess replication originating from R-loops.

The origins of replication for cSDR (*oriK*s) in *rnhA* null mutants are mostly found within or close to the *ter* region where bi-directional replication initiated at *oriC* normally terminates [54]. Thus, the origin to terminus (*oriC/ter*) ratio, is expected to be lowered by the occurrence of cSDR. This is indeed what we found for the *rnhA* null mutant (Fig. S10, RFM443 vs RFM430 *rnhA::cam*). The *ori/ter* ratio was also similarly reduced in the *topA* null mutant, thus supporting the occurrence of cSDR in the absence of topo I (Fig. S10, RFM475).

Several of our *kan*$^r$ insertion mutants were found to reduce the expression of the *holC* gene (Valentine and Drolet, manuscript in preparation). In a previous study, *kan*$^r$ insertion mutants that reduced the expression of the *holC* gene were also found to suppress the growth defect of a *dnaAcos* strain [65]. The *holC* gene encodes the χ subunit of the

clamp loader, the replicative polymerase in *E. coli* [66]. The χ subunit interacts with SSB and this interaction was recently shown to play an important role in replisome establishment and maintenance [67]. We tested the *holC2*::*aph* mutation for its ability to suppress phenotypes of cells lacking type 1A topos. For this purpose, a *topA topB gyrB*(Ts) *holC2*::*aph* strain, carrying the *topA20*::Tn*10* allele, was constructed. The *holC2*::*aph* mutation was shown to slightly correct the growth defect of cells lacking type 1A topos activity (Fig. 7b and c, 30 and 24ºC respectively, RFM445 *topB holC topA* vs RFM445 *topB topA*). Both cell length and the amount of DNA were also slightly reduced (Fig. 7a). The fact that *holC* mutations by themselves can cause filamentation and chromosome segregation defects [67], may explain why the *holC2*::*aph* mutation only partially corrected the phenotypes of the *topA topB gyrB*(Ts) strain.

The *holC2*::*aph* mutation also partially corrected the growth defect of our *topA* null mutant (Fig. 2b, 24ºC, 48 h; RFM475 vs RFM475 *holC*). Moreover, in rifampicin run-out experiments, we found that replication was not well regulated in the *topA* null mutant carrying the *holC2*::*aph* mutation, as peaks reflecting 1, 2, 3, or 4 chromosomes were clearly observed (Fig. S11, RFM475 vs RFM475 *holC2*::*aph*). This result supports the hypothesis that the χ subunit of pol III plays a role in replication initiation [67] and therefore suggests that initiation from *oriC* could also be problematic in cells lacking both type 1A topos.

# Discussion

### *E. coli* type 1A topos and RecQ

As stated in the introduction, the strand passage activity of *E. coli* topo III, but not topo I, was shown to be strongly stimulated by RecQ *in vitro* [35-37]. This would suggest that *E. coli* topo III and RecQ can act together to maintain the stability of the genome, as shown in eukaryotic cells [51]. However, no clear evidence for such a role of topo III has been reported in *E. coli*. Recent experimental evidence rather points to a role for topo III in chromosome segregation related to replication and independent of RecQ ([33,34]; this work). In fact, the data presented here suggest that topo I, not topo III, is the primary type 1A topo acting with RecQ in *E. coli*. Indeed, the strong chromosome segregation and growth defects of *topA* null cells at low temperatures were shown to be partially corrected by deleting *recQ* or *recA*, independent of the RecFOR pathway. This is consistent with RecQ processing RecA-dependent recombination intermediates in such a way that they can only be resolved by a type 1A topo, as is the case in eukaryotic cells. In this context, topo III overproduction would substitute for topo I and perform the resolution, thus meaning that topo III can also perform this reaction *in vivo*, at least when it is overproduced. This would also explain why overproducing topo III had no effects on the growth of *topA* null cells lacking either *recA* or *recQ*. Furthermore, we found that topo III overproduction does not correct the replication initiation phenotype of *topA* null cells as shown by rifampicin run-out experiments (not shown).

Alternatively, in the absence of *topA,* DNA substrates for topo I may accumulate and some of them could be processed by topo III, thus leading to the depletion of this very low abundant protein. This situation would lead to the accumulation of RecQ-processed recombination intermediates, if topo III normally resolves them. However, we think that this is unlikely for two reasons. Firstly, if topo III was the type 1A topo acting with RecQ in *E. coli*, we could predict that any mutation increasing replication from *oriC* (as shown here for the *topA* null mutation) would necessitate the action of topo III to resolve RecQ-

processed recombination intermediates. However, we found that deleting *topB* from a *seqA gyrB*(Ts) strain had no effects on its growth (37ºC and below were tested; not shown). SeqA proteins sequester freshly replicated *oriC* sequences to avoid over-initiation and asynchrony [68]. Thus, if indeed a type 1A topo is required to act with RecQ in cells over-initiating from *oriC*, this enzyme is not topo III, but topo I. Secondly, whereas a *topA recQ* strain grow very well, deleting *topB* make this strain very sick with phenotypes identical to those of *topA topB* null cells. If *recQ* was acting with *topB*, then deleting *topB* should have had no effect on the growth of the *topA recQ* strain. Thus, altogether our data suggest that topo I is the primary type 1A enzyme acting with RecQ in *E. coli.*

Despite the previously observed lack of stimulation of topo I activity by RecQ *in vitro*, we still believe that these two proteins can functionally interact. Indeed, it may be that the optimal experimental conditions and/or the appropriate substrate for their functional interaction have not yet been well defined. Alternatively or additionally, the much higher abundance of topo I *in vivo* as compared to topo III may compensate for its lower level of activity with RecQ. In fact, the finding that either *E. coli* topo I expression or a *SGS1* mutation could compensate for the absence of Top3 in *S. cerevisiae* [38-40], support the assumption that *E. coli* topo I can act with RecQ *in vivo*. Moreover, in an *in vitro* system for DHJs resolution by BLM helicase with a type 1A topo, *E. coli* topo I was shown to efficiently substitute for human topo IIIα [69]. Hsieh and co-workers have recently obtained experimental evidence for their "unravel and unlink" model whereby BLM first melts a DNA region to which RPA protein binds and topo IIIα acts to resolve a DHJ [1,43]. Indeed, a topo IIIα mutant unable to physically interact with BLM was shown to partially resolve a DHJ in the presence of RPA, thus suggesting that the functions of the two proteins may be separated [43]. A similar model might also be proposed for RecQ acting with topo I, the activity of which can be stimulated by SSB [70], as the two proteins do not physically interact.

### *E. coli* type 1A topos in replication

In *E. coli*, replication initiated at *oriC* is tightly regulated so that it occurs once and only once per cell cycle [57]. This process is synchronized with the "initiation mass". DNA supercoiling is among the many elements, including DnaA that are required for replication initiation at *oriC*. Indeed, *in vitro* replication initiation requires that the *oriC* plasmid be negatively supercoiled [71]. *In vivo*, deleting *topA* was found to correct the thermo-sensitive growth of a *dnaA*(Ts) mutant [27] and altering gyrase supercoiling activity inhibited replication initiation from *oriC* [72]. Moreover, we have recently shown that a *topA* deletion could correct the replication initiation defect of a strain defective for gyrase supercoiling activity [34]. Interestingly, in a screen to isolate DnaA inhibitors, a compound was recently found to rescue a *dnaAcos* mutant from lethal hyperinitiation by targeting gyrase [73]. Thus, *in vitro* and *in vivo* data demonstrate that negative DNA supercoiling is required for replication initiation from *oriC*.

The recent determination of the crystal structure of a truncated DnaA ortholog in complex with ssDNA supports a model whereby DnaA opens the *oriC* region by a direct ATP-dependent stretching mechanism [74]. This work provides the strongest evidence to date for a direct participation of DnaA in DNA melting at *oriC*, and is fully compatible with other elements, such as DNA supercoiling, also playing a role in this process. In a recent biochemical study, DNA fragments containing at least the left portion of *oriC* up to I1 or I2 (Fig. 4a) were shown to be required for DnaA-ATP binding to ssDUE in the absence of torsional stress [75]. This result is totally consistent with our finding that an *oriC* region lacking these I1 and I2 sequences (*oriC15*::*aph*) is functional in a *topA* null mutant, where the negative supercoiling level is elevated, but not functional in an isogenic *topA*$^+$ strain. Thus, our results, together with those reported in the two studies mentioned above, may suggest that DNA supercoiling plays an important regulatory role at *oriC*.

The *de novo* synthesis of DnaA is required for a cell to obtain the appropriate level of DnaA-ATP needed for replication initiation at *oriC* [57]. In this way, replication initiation can be adjusted to the growth rate. We speculate that in *topA* null cells, because of the excess negative supercoiling at *oriC*, less DnaA-ATP would be required for replication

initiation at *oriC*. This, in turn, would cause more frequent replication initiation and, therefore, over-replication. The level of negative supercoiling and thus the frequency of replication initiation may be especially high at lower temperatures (e.g. 24°C) in *topA* null cells, where topo IV activity is also inhibited. The high number of replication forks travelling on the chromosome may increase the frequency of both fork collisions between themselves and fork collisions with various obstacles such as RNA polymerases [76]. This could lead to genomic instability and hyper-recombination with the generation of RecQ-processed recombination intermediates requiring a type 1A topo for resolution. In addition, the excess negative supercoiling, by promoting DNA melting, may render the chromosome more susceptible to DNA damage.

The important role played by topo I in replication initiation at *oriC* could explain why RNase HI overproduction sometimes fails to complement the growth of *topA* null cells [47,50,77]. We have previously shown that upon a temperature downshift (from 37 to 28°C), the growth of our *topA* null *gyrB*(Ts) strain was transiently inhibited, because of the failure to accumulate full-length RNAs [61]. As a consequence, protein synthesis (likely including the synthesis of DnaA) and replication initiation, were also transiently inhibited [61]. Moreover, growth eventually resumed, albeit at a lower rate compared to the *topA* null *gyrB*(Ts) strain overproducing RNase HI that did not suffer from a transient inhibition. Thus, RNase HI overproduction by stimulating protein synthesis (and the accumulation of DnaA) also stimulated replication initiation from *oriC* in *topA* null mutants. In this context, the introduction of a *topA* null mutation in a wild-type strain overproducing RNase HI could rapidly trigger over-replication, thus causing chromosome segregation defects that would preclude the survival of the transductants.

When *topB* was deleted from a *topA* null mutant, a new growth inhibitory phenotype, again related to replication, appeared at temperatures where the *oriC*-related phenotype was attenuated. Our data suggest that this major phenotype in the absence of type 1A topos is related to replication from R-loops (cSDR). This is consistent with a major role of topo I in the inhibition of R-loop formation and with the identification of topo I, like RNase HI [78], as a specificity factor to inhibit replication initiation at sites other than *oriC* (e.g. R-loops), in an *in vitro* system [28]. Thus, although the strong phenotype expressed

such as extensive cell filamentation, unsegregated nucleoids and growth inhibition, is triggered by deleting *topB*, cSDR is probably also activated in our single *topA* mutant. This is supported by the fact that the *dnaT18*::*aph* mutation improved the growth of our *topA* mutant and by the finding that, as was the case in an *rnhA* null mutant, the *ori/ter* ratio was lower in this *topA* mutant as compared to a wild-type strain. However, even if cSDR is activated in *topA* null mutants, the *oriC*/DnaA system is still required in these cells to replicate the chromosome. A similar situation has been described for *recG* mutants, in which cSDR is also activated but cannot support replication of the whole chromosome [79,80].

As the strong phenotype is due to the simultaneous absence of both type 1A topos, it is likely related to similar functions performed by the two enzymes. We have previously shown that an R-loop was a hot-spot for topo III activity *in vitro* [47]. By acting on an R-looped plasmid, topo III was shown to destabilize the R-loop. As topo III can travel with the replication fork [35], it could possibly act by destabilizing R-loops blocking the progression of the replication forks. However, if topo III was playing an important role in R-loop removal, a *topB* strain lacking *rnhA* should be very sick as is the case for a *topA* mutant without *rnhA*. Whereas *topA rnhA* mutants display phenotypes similar to *topA topB* mutants ([17] and Fig. S9), *topB rnhA* mutants grow very well and show no obvious phenotypes (Usongo and Drolet, unpublished results). It may be that topo I is the major enzyme involved in R-loop inhibition and that another function of topo I (e.g. in acting with RecQ) is necessary when R-loops accumulate. Still, in this scenario, it is unclear why deleting *topB* would have such a strong effect on a *topA* null mutant.

One function of topo III when the enzyme is not overproduced, appears to be DNA decatenation during replication to allow chromosome segregation [33,34]. Topo III likely acts at the replication fork where it physically interacts with SSB protein [35]. Another ssDNA decatenation activity appears to be provided by topo I with the help of RecQ as suggested from the results of our study. A ssDNA decatenation activity, either provided by topo I or III, may be required in the cell especially when the chromosomal DNA is over-replicated from either *oriC* or R-loops due to excess negative supercoiling, as is likely the case in our *topA* mutant at 30$^{o}$C. Furthermore, this ssDNA decatenation activity might be

especially required to resolve convergent replication forks, in order to prevent over-replication when forks collide [81] as proposed [82]. Such unnecessary replication, named "pathological replication", has been detected in *recG* cells suffering from SDR, mostly because replication forks could then meet outside the *ter* region [80,83,84]. Interestingly, excess negative supercoiling in a *topA* mutant has been shown to reduce the efficacy of Tus-mediated arrest of replication forks at *ter* sites [85] and more recently a double *topA recG* mutant could not be constructed unless topo III was overproduced [77]. Thus, although our results suggest that type 1A topos are required to prevent deleterious over-replication, more work is needed to better establish at which steps they act.

# Materials and Methods

### Bacterial strains and plasmids

Bacterial strains used in this study are all derivatives of *E. coli* K12 and are listed in Table S1. Details on their constructions as well as the list of plasmids used in this study are also given in Table S1. Transductions with P1*vir* were performed as described previously [34]. PCR was used to confirm that the expected gene transfer occurred in the selected transductants.

### Insertional mutagenesis

Insertional mutagenesis with pRL27 was performed in a *topA rnhA gyrB*(Ts) strain and will be described in details elsewhere (Usongo and Drolet, manuscript in preparation). Briefly, pRL27 carries a hyperactive Tn5 transposase gene under the control of the *tetA* promoter and an insertional cassette with a kanamycin resistance gene (*aph*) and a *pir*-dependent origin (*oriR6K*), bracketed by Tn5 inverted repeats [86]. Following electroporation of pRL27 in a *pir*- background, the *kan*$^r$ cassette inserts randomly into the chromosome. A *topA rnhA gyrB* (Ts)/pBAD18*rnhA* strain was electroporated with pRL27 and plated on LB containing 25 μg/ml kanamycin at 40ºC, to select for suppressors that grew in the absence of arabinose (no RNase HI produced). At this temperature, the strain does not grow because of extensive inhibition of the supercoiling activity of gyrase [17], combined with over-replication ([34] and see below). P1*vir* was grown on the *kan*$^r$ clones that re-grew at 40ºC and each phage lysate was used to infect a *topA rnhA gyrB* (Ts)/pPH1243 strain, that normally grows only in the presence of IPTG, to overproduce topo III from pPH1243 [17]. Transductants were selected on LB plates containing IPTG and kanamycin (50 μg/ml) at 37ºC. Transductants that re-grew in the absence of IPTG were selected for further characterization. Four of the insertion mutants, described in Fig. 4 and S8, were used in the present study.

**Spot tests**

Cells from glycerol stock were resuspended in LB to obtain an $OD_{600}$ of 0.6. Five µl of 10-fold serial dilutions were then spotted on LB plates incubated at the indicated temperatures. The experiments were performed with cells from glycerol stock to minimize the chance of selecting cells with compensatory mutations. However, we eventually found that similar results were obtained whether the cells were from glycerol stock or from overnight liquid cultures (not shown).

**Microscopy**

Cells were grown overnight at 37ºC in liquid LB medium supplemented with the appropriate antibiotics. Overnight cultures were diluted in LB medium to obtain an $OD_{600}$ of 0.01 and grown at the indicated temperature to an $OD_{600}$ of 0.8. The cells were recovered and prepared for microscopy as previously described [17]. Pictures (fluorescence (DAPI) and DIC) were randomly taken with a LSM 510 Meta confocal microscope from Zeiss. The images were processed using Adobe Photoshop. Representative images are shown both in the Results and Supporting Information sections.

**Flow cytometry**

The procedure for flow cytometry in rifampicin run-out experiments with cells grown in M9 medium has been described [34]. The DNA/mass ratio was calculated has previously reported [34].

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# TABLES AND FIGURES

**Table S1** *E. coli* strains and plasmids used

| Name | Genotype | Reference or Source |
|---|---|---|
| MA251 | *gyrB221* (Cou$^r$) *gyrB203*(Ts) *zie-3163*::Tn*10kan topA20*::Tn*10* | [1] |
| CL581 | *thyA36*, *deoC2*, *IN(rrnD-rrnE)I, rph?*, *recQ6215* (sub cat 883 for cdn 19-606) *recQ6215::cam* | Justin Courcelle |
| CT150 | RFM475 *recQ6215::cam* | RFM475 x P1(CL581) |
| CT170 | RFM475 *ΔtopB::kan* | [2] |
| JE35 | PH379 *dnaA46*(Ts) *tnaA*::Tn*10* | PH379 x P1(MD48) |
| JE36 | JE35 *rne59*::*aph* | This work[a] |
| JE119 | MD48 *rne59*::*aph* | This work[a] |
| JW0461-2 | *ΔrecR776::kan* | [3] |
| JW1752-1 | *ΔtopB761::kan* | [3] |
| JW2549-1 | *ΔrecO737::kan* | [3] |
| JW2860-1 | *ΔrecJ743::kan* | [3] |
| KA441 | *ilv thyA thr tyrA*(Am) *trpE9829*(Am) *metE deo supF6*(Ts) *dnaAcos tna*::Tn*10* | [4] |
| MD48 | MC4100 *dnaA46*(Ts) *tnaA*::Tn*10* | Lab collection |
| PH379 | *rnhA ::cam* | [5] |
| RFM443 | *rpsL galK2* Δ*lac74* | [6] |
| RFM445 | *rpsL galK2 gyrB221* (Cou$^r$) *gyrB203*(Ts) Δ*lac74* | [6] |
| RFM475 | *rpsL galK2 gyrB221* (Cou$^r$) *gyrB203*(Ts) Δ*lac74* Δ*(topA cysB)204* | [6] |

| | | |
|---|---|---|
| SB265 | RFM475 Δ*recA306 srlR301*::Tn*10* | RFM475 x P1(VU29) |
| VU29 | RFM443 Δ*recA306 srlR301*::Tn*10* | Lab collection |
| VU118 | RFM475 pPH1243 | This work |
| VU129 | VU118 *rnhA::cam* | VU118 x P1(PH379) |
| VU148 | VU129 *dnaT18::aph* | This work[a] |
| VU155 | RFM475 *oriC15::aph* | This work[a] |
| VU176 | RFM475 *holC2::aph* | This work[a] |
| VU188 | RFM475 *dnaT18::aph* | This work[a] |
| VU194 | KA441 *oriC15::aph* | This work[a] |
| VU200 | JE35 *dnaT18::aph* | This work[a] |
| VU204 | MD48 *dnaT18::aph* | This work[a] |
| VU205 | CT150 Δ*topB::kan* | CT150 x P1(DM4100 Δ*topB::kan)* |
| | CT170 Δ*recA306 srlR301*::Tn*10* | |
| VU243 | RFM475 pSK760 | CT170 x P1(VU29) |
| VU287 | RFM475 pSK762c | This work |
| VU299 | CT170 pSK760 | This work |
| VU306 | CT170 pSK762c | This work |
| VU333 | RFM475 *ΔrecJ743::kan* | This work |
| VU349 | VU205 pSK760 | RFM475 x P1(JW2860-1) |
| VU363 | VU205 pSK762c | This work |
| VU365 | VU243 pSK760 | This work |
| VU375 | VU243 pSK762c | This work |
| VU379 | RFM445 *ΔtopB761::kan* | This work |
| VU403 | RFM445 *ΔtopB* | RFM445 x P1( JW1752-1) |
| VU409 | VU409 *dnaT18::aph* | VU403, *kan* removed by pCP20 |
| | VU409 *rne59*::*aph* | [7] |
| VU414 | VU409 *topA20*::Tn*10* | This work[a] |
| VU416 | VU421 pSK760 | This work[a] |
| VU421 | VU421 pSK762c | VU409 x P1(MA251) |

| | | |
|---|---|---|
| VU422 | VU414 *topA20*::Tn*10* | This work |
| VU425 | RFM475 *ΔrecO737::kan* | This work |
| VU441 | RFM475 *ΔrecR776::kan* | VU414 x P1(MA251) |
| VU454 | CT150 pPH1243 | RFM475 x P1(JW2549-1) |
| VU458 | VU409 *holC2::aph* | RFM475 x P1(JW0461-2) |
| VU464 | VU468 *topA20*::Tn*10* | This work |
| VU468 | VU416 *topA20*::Tn*10* | This work[a] |
| VU469 | SB265 pPH1243 | VU468 x P1(MA251) |
| VU473 | *topB* under the control of | VU416 x P1(MA251) |
| VU479 | IPTG-inducible promoter | This work |
| pPH1243 | *rnhA* gene with its own promoter | [8] |
| | like pSK760 but *rnhA* is mutated and | |
| pSK760 | inactive | [6] |
| pSK762c | | [6] |

[a] The P1*vir* phage lysates used to transfer the various insertion mutants described in this study were obtained from the original suppressed clones obtained at 40°C (see Material and Methods)

## Supplemental References


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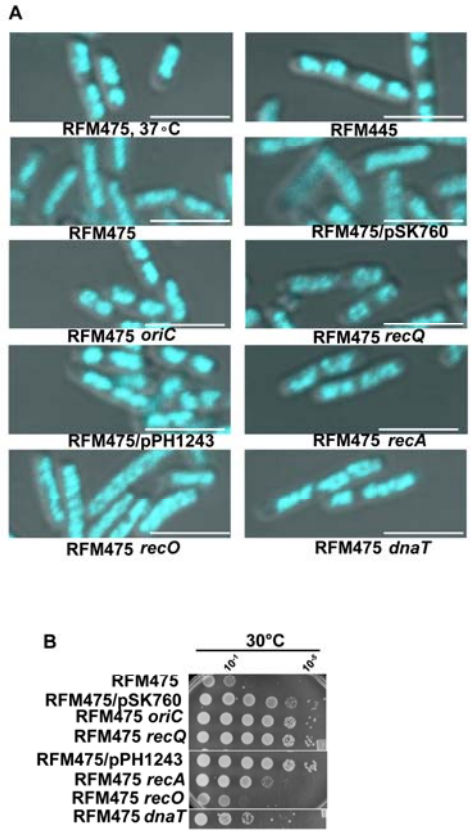



**Figure 1. Growth and chromosome segregation defects in a *topA gyrB*(Ts) strain at 30°C.** (a) Representative superimposed images of DIC and fluorescence pictures of DAPI-stained cells grown at 30°C, unless otherwise indicated, as described in Materials and Methods. Size bars are 5 µm. Additional images are shown in Fig. S1. (b) Spot tests at 30°C. The LB plates were incubated for 24 h. The strains used are all derivatives of RFM475 (Δ*topA gyrB*(Ts)) except RFM445 (*gyrB*(Ts)). They are: VU287 (RFM475/pSK760), VU155 (RFM475 *oriC*), CT150 (RFM475 *recQ*), VU118 (RFM475/pPH1243), SB265 (RFM475 *recA*), VU469 (RFM475 *recO*) and VU188 (RFM475 *dnaT*). pSK760 carries the *rnhA* gene for RNase HI overproduction. Cells carrying pPH1243 were grown in the presence of IPTG to overproduce topo III.

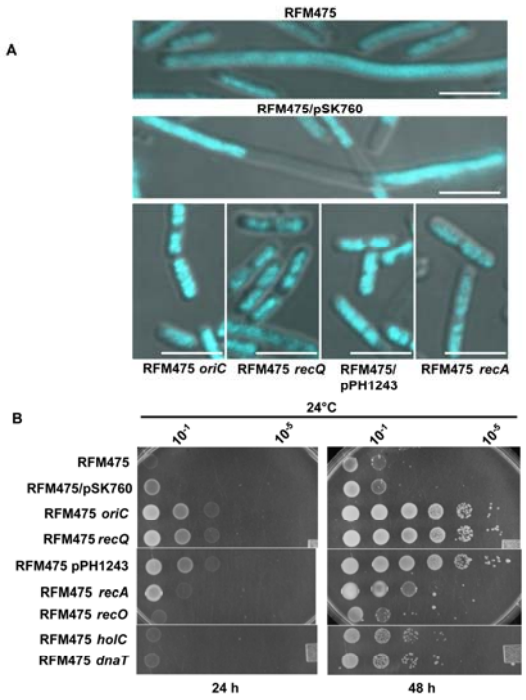



**Figure 2. Growth and chromosome segregation defects in *topA gyrB*(Ts) strains at 24ºC.** (a) Representative superimposed images of DIC and fluorescence pictures of DAPI-stained cells grown at 24ºC as described in Materials and Methods. Size bars are 5 µm. Additional images are shown in Fig. S2. (b) Spot tests at 24ºC. Pictures of LB plates after 24 and 48 h of incubation as indicated. The strains used are all derivatives of RFM475 (Δ*topA gyrB (*Ts)). They are: VU287 (RFM475/pSK760), VU155 (RFM475 *oriC*), CT150 (RFM475 *recQ*), VU118 (RFM475/pPH1243), SB265 (RFM475 *recA*), VU469 (RFM475 *recO*), VU188 (RFM475 *dnaT*) and VU176 (RFM475 *holC*). pSK760 carries the *rnhA* gene for RNase HI overproduction. Cells carrying pPH1243 were grown in the presence of IPTG to overproduce topo III.

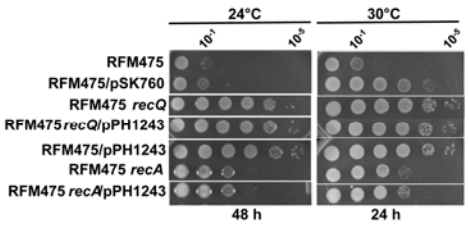
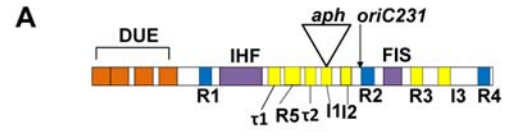
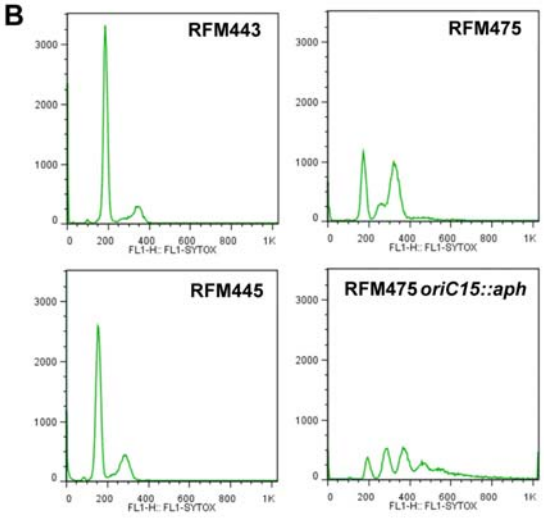



**Figure 3. *recA* and *recQ* deletions are epistatic to *topB* overexpression in correcting the growth defect of a *topA gyrB*(Ts) strain.** Spot tests at 24 and 30ºC as indicated. The LB plates were incubated at 24 and 30ºC for respectively 48 and 24 h. The strains used are all derivatives of RFM475 (*topA gyrB* (Ts)). They are: VU287 (RFM475/pSK760), CT150 (RFM475 *recQ*), VU464 (CT150/pPH1243), VU118 (RFM475/pPH1243), SB265 (RFM475 *recA*), and VU479 (SB265/pPH1243). pSK760 carries the *rnhA* gene for RNase HI overproduction. Cells carrying pPH1243 were grown in the presence of IPTG to overproduce topo III.

**Figure 4. Replication initiation asynchrony and reduced DNA/mass ratio conferred by the *oriC15*::*aph* suppressor mutation isolated from a *topA rnhA gyrB*(Ts) strain.** (a) Schematic representation of the minimal *oriC* region (245 bp) with its regulatory elements. DUE is the DNA unwinding element with its AT-cluster and 13-mer repeats L, M, and R (orange). DnaA binding sites: R1, R2 and R4 are high affinity sites (blue) whereas R3, R5, I1-3 and τ1-2 are low affinity sites (yellow). I1-3 and τ1-2 preferentially bind DnaA-ATP. IHF and FIS binding sites are also shown. For more details see [57]. *aph* indicates the insertion site of the *kan*$^{r}$ cassette in our *oriC15::aph* insertion mutant (position 142 in the 245 bp *oriC* region). The *oriC231* allele of Stepankiw *et al*. [56] spanning the left portion of *oriC* up to the arrow is shown for comparison (position 163 in the 245 bp *oriC* region). (b) Rifampicin run-out experiments for flow cytometry analysis were performed as described in Materials and Methods. Cells were grown in M9 minimal medium. (c) DNA/mass ratios were calculated as described in Material and Methods from three independent flow cytometry experiments. The strains used were: RFM443 (wild-type), RFM445 (*gyrB* (Ts)), RFM475 (*topA gyrB* (Ts)) and VU155 (RFM475 *oriC15::aph*).


A
DUE
IHF
aph
oriC231
FIS
R1
τ1
R5
τ2
I1
I2
R2
R3
I3
R4


B
RFM443
RFM475
RFM445
RFM475 oriC15::aph
FL1-H: FL1-SYTOX

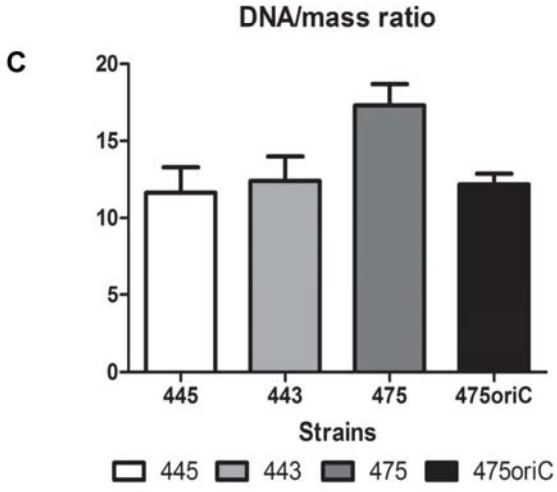
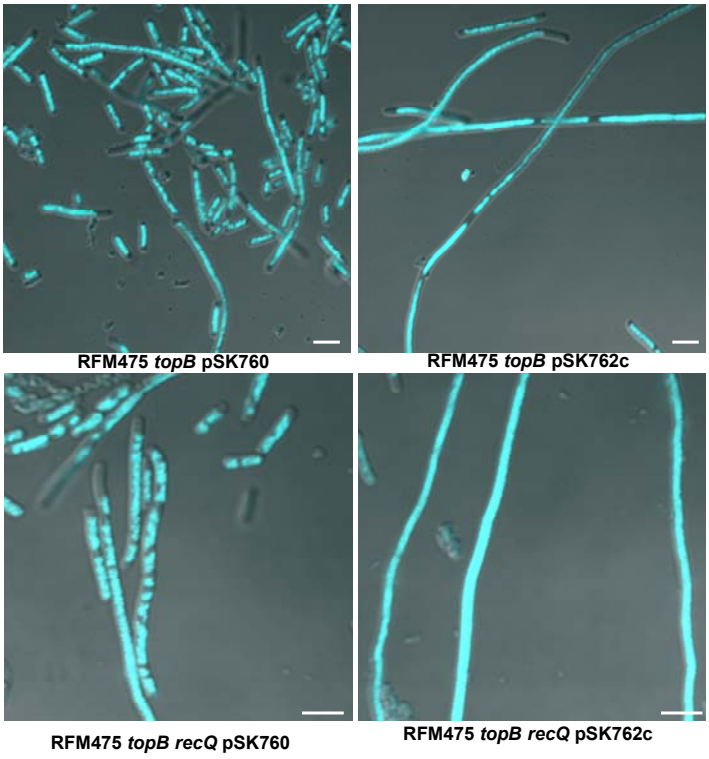

C
DNA/mass ratio
20
15
10
5
0
445
443
475
475oriC
Strains
445
443
475
475oriC

**Figure 5. The growth and chromosome segregation defects in cells lacking type 1A topos are partially corrected by overproducing RNase HI or by deleting *recA* but not *recQ*.** (a) Representative superimposed images of DIC and fluorescence pictures of DAPI-stained cells grown at 30ºC as described in Materials and Methods. Size bars are 5 μm. (b) Spot tests at 30ºC. The LB plate was incubated for 24 h. The strains used are all derivative of RFM475 (Δ*topA, gyrB* (Ts)). They are: VU306 (RFM475 *topB*/pSK760), VU333 (RFM475 *topB*/pSK762c), VU363 (RFM475 *topB recQ*/pSK760), VU365 (RFM475 *topB recQ*/pSK762c), VU375 (RFM475 *topB recA*/pSK760) and VU379 (RFM475 *topB recA*/pSK762c). pSK760 carries the *rnhA* gene for RNase HI overproduction, whereas pSK762C carries a mutated and inactive *rnhA gene*.

**A**



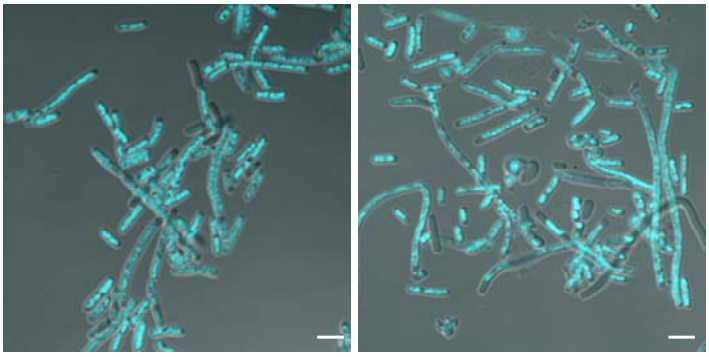
RFM475 *topB* pSK760

RFM475 *topB* pSK762c

RFM475 *topB recQ* pSK760

RFM475 *topB recQ* pSK762c

A



RFM475 *topB recA* pSK760

RFM475 *topB recA* pSK762c

B

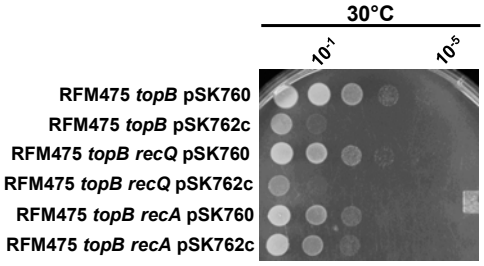


**Figure 6. Both the *dnaT18*::*aph* and *rne59*::*aph* suppressor mutations isolated from a *topA rnhA gyrB*(Ts) strain inhibit cSDR in an *rnhA* strain.** (a) Model for constitutive stable DNA replication (cSDR) [54,60]. R-loop forms during transcription when the nascent RNA hybridizes with the template DNA strand behind the moving RNA polymerase. Both transcription-induced negative supercoiling and RecA protein promote R-loop formation. DNA pol I synthesizes DNA from the 3' end of the hybridized RNA for primosome (PriA-dependent) assembly. Eventually, the primosome allows the assembly of two replisomes for bidirectional replication. The proteins that are included in the present study are shown in red: topo I relaxes transcription-induced negative supercoiling; RecA promotes the hybridization of the template DNA strand with the nascent RNA [87,88]; RNase HI degrades the RNA of the R-loop; RNase E may inhibit R-loop formation by degrading the nascent RNA; DnaT may play a role in cSDR via the primosome. (b) A map of the *E. coli* chromosome showing the normal origin of replication (*oriC*), the putative cSDR origins of replication (*oriK*, [54]) and two of the ten *ter* sites, with *terC* believed to be a site where many convergent replication forks meet [82]. (c) and (d). Spot tests. The LB plates were incubated for 24 h, at 30 or 42ºC as indicated. The strains used were: MD48 (*dnaA46*(Ts)), JE35 (*rnhA dnaA46*(Ts)), VU204 (*dnaA46*(Ts), *dnaT*), VU200 (*rnhA dnaA46*(Ts) *dnaT*), JE36 (*rnhA dnaA46*(Ts) *rne*) and JE119 (*dnaA46*(Ts) *rne*). At 42ºC, the few colonies of strain MD48 (at $10^0$ and $10^{-1}$) were made of cells that have acquired compensatory mutations, as they grew robustly upon restreaking them at the same temperature.

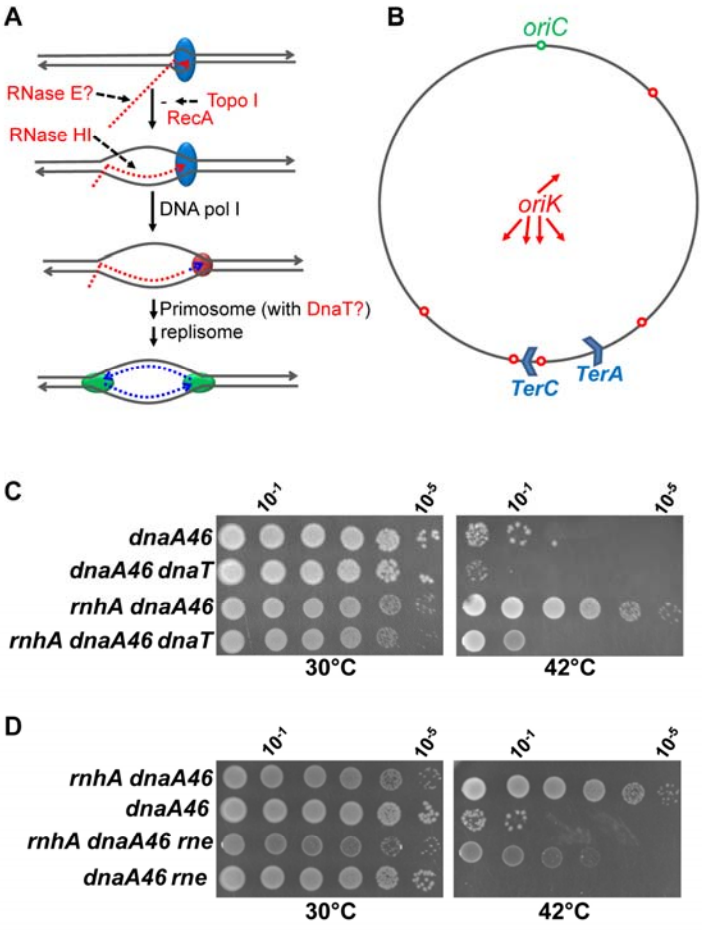
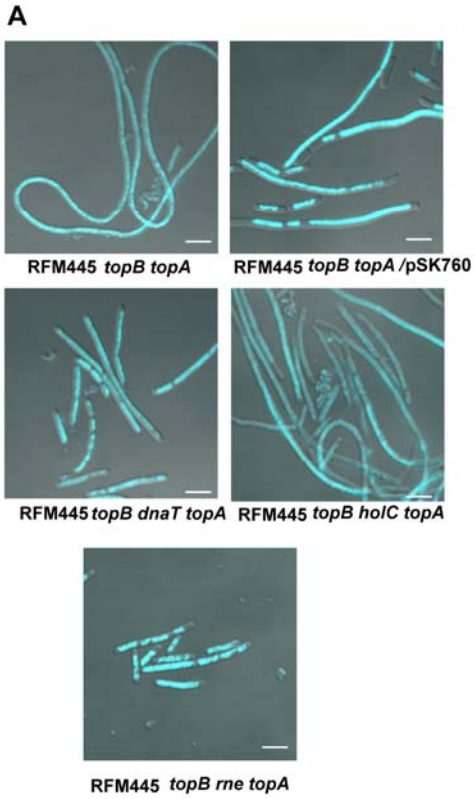

A
RNase E?
Topo I
RecA
RNase HI
DNA pol I
Primosome (with DnaT?)
replisome
B
oriC
oriK
TerC
TerA
C
10-1
10-5
dnaA46
dnaA46 dnaT
rnhA dnaA46
rnhA dnaA46 dnaT
30°C
42°C
D
rnhA dnaA46
dnaA46
rnhA dnaA46 rne
dnaA46 rne
30°C
42°C

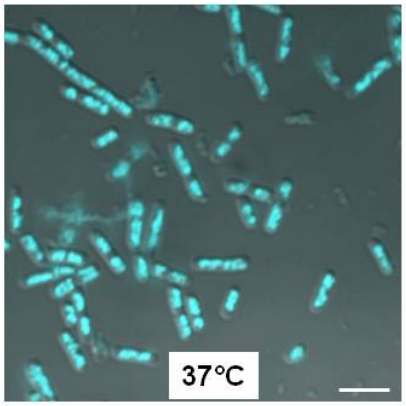
**Figure 7. The growth and chromosome segregation defects in cells lacking type 1A topos are partially corrected by *dnaT18*::*aph*, *rne59*::*aph* and *holC2*::*aph* suppressor mutations isolated from a *topA rnhA gyrB*(Ts) strain.** Representative superimposed images of DIC and fluorescence pictures of DAPI-stained cells grown at 30ºC as described in Materials and Methods. Size bars are 5 μm. (b) Spot tests at 30ºC (a), 24ºC (b) and 37ºC (c). The LB plates were incubated for the indicated period of time. The strains used are all derivative RFM445 *topB* (strain VU409: *gyrB* (Ts), Δ*topB*). They are: VU421 (RFM445 *topB topA20*::Tn*10*), VU422 (RFM445 *topB topA20*::Tn*10*/pSK760), VU425 (RFM445 *topB topA20*::Tn*10*/pSK762c), VU441 (RFM445 *topB dnaT topA20*::Tn*10*), VU469 (RFM445 *topB holC topA20*::Tn*10*) VU473 (RFM445 *topB rne topA20*::Tn*10*). pSK760 carries the *rnhA* gene for RNase HI overproduction, whereas pSK762C carries a mutated and inactive *rnhA gene*.


A
RFM445 topB topA
RFM445 topB topA /pSK760
RFM445 topB dnaT topA
RFM445 topB holC topA
RFM445 topB rne topA
B
30°C
RFM445 topB topA
RFM445 topB topA / pSK760
RFM445 topB dnaT topA
RFM445 topB holC topA
RFM445 topB rne topA
24 h
48 h
C
24°C
RFM445 topB topA
RFM445topB topA /pSK760
RFM445 topB topA pSK762c
RFM445 topB dnaT
RFM445 topB holC
48 h
D
37°C
RFM445 topB topA /pSK760
RFM445 topB topA
RFM445 topB dnaT topA
24 h

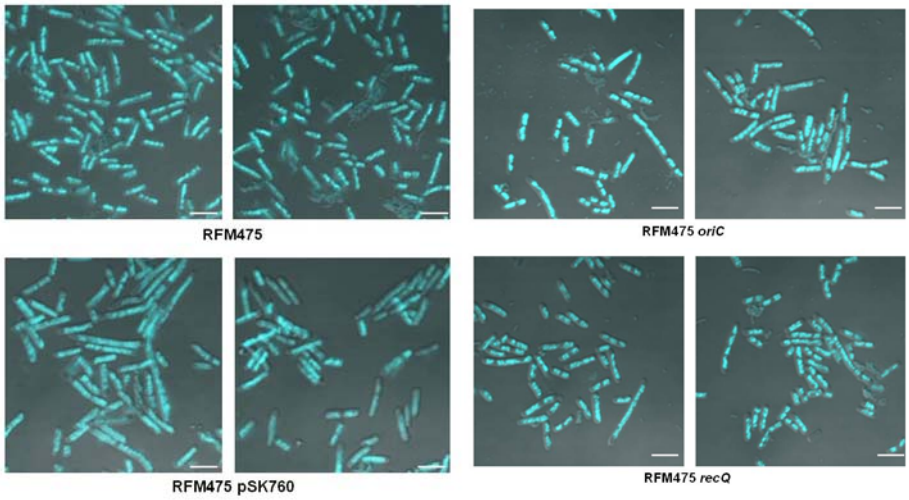
# Supporting information

**Figure S1. Chromosome segregation defects in a *topA gyrB*(Ts) strain at 30ºC.** Superimposed images of DIC and fluorescence pictures of DAPI-stained cells grown at 30ºC, unless otherwise indicated, as described in Materials and Methods. Size bars are 5 μm. The strains used are all derivatives of RFM475 (Δ*topA gyrB* (Ts)) except RFM445 (*gyrB* (Ts)). They are: VU287 (RFM475/pSK760), VU155 (RFM475 *oriC*), CT150 (RFM475 *recQ*), VU118 (RFM475/pPH1243), SB265 (RFM475 *recA*), VU469 (RFM475 *recO*) and VU148 (RFM475 *dnaT*). pSK760 carries the *rnhA* gene for RNase HI overproduction. Cells carrying pPH1243 where grown in the presence of IPTG to overproduce topo III.

Fig. S1




RFM475


RFM475
RFM475 oriC
RFM475 pSK760
RFM475 recQ

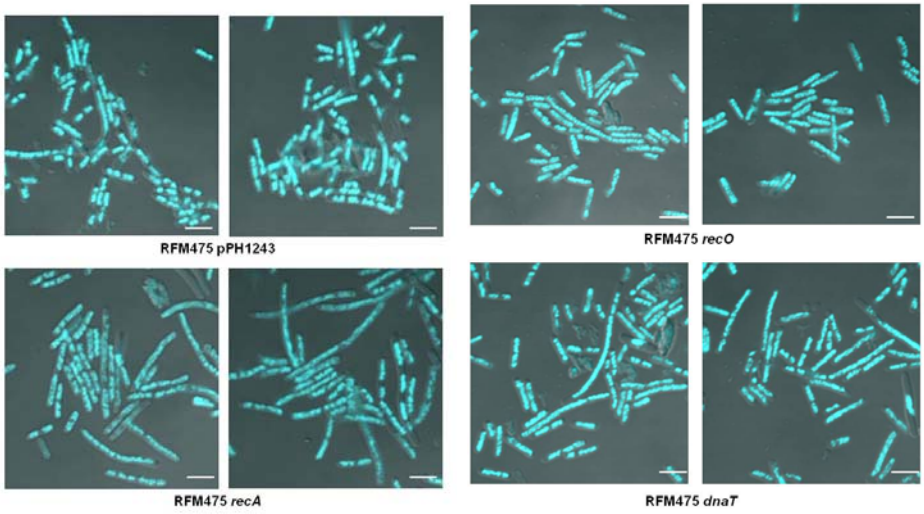
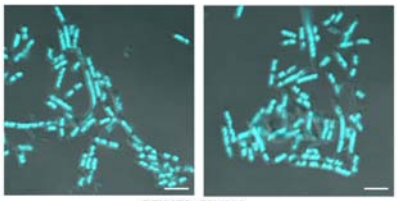
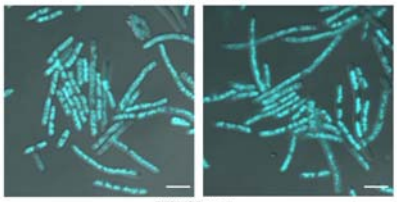

RFM475 pPH1243
RFM475 recO
RFM475 recA
RFM475 dnaT

**Figure S2. Chromosome segregation defects in a *topA gyrB*(Ts) strain at 24ºC.** Superimposed images of DIC and fluorescence pictures of DAPI-stained cells grown at 24ºC, unless otherwise indicated, as described in Materials and Methods. Size bars are 5 μm. The strains used are all derivatives of RFM475 (Δ*topA gyrB (*Ts)). They are: VU287 (RFM475/pSK760), VU155 (RFM475 *oriC*), CT150 (RFM475 *recQ*), VU118 (RFM475/pPH1243) and SB265 (RFM475 *recA*). pSK760 carries the *rnhA* gene for RNase HI overproduction. Cells carrying pPH1243 where grown in the presence of IPTG to overproduce topo III.

**Fig. S2**



RFM475 pPH1243



RFM475 *recA*

Fig. S3

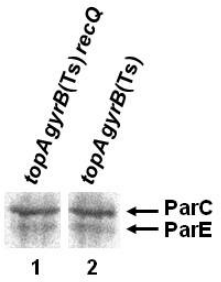
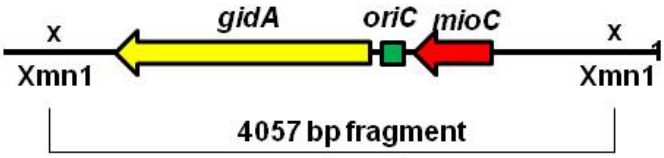



**Figure S3. Topo IV is not overproduced following the deletion of *recQ* in strain RFM475.** Cells were grown overnight on LB plates at 37°C. Aliquots were recovered for Western blotting using anti-ParC and anti-ParE antibodies as described by Usongo *et al.* (2008). Strains used are RFM475 *(topA gyrB*( Ts)) and CT150 (RFM475 *recQ*). The result shown here is representative of three independent experiments. (Usongo V, Tanguay C, Nolent F, Bessong JE, Drolet M (2008) Interplay between type 1A topoisomerases and gyrase in chromosome segregation in *Escherichia coli*. Mol Micro 195:1758-1768.

**Figure S4. Southern blot experiment showing the successful transfer of the *oriC*::Tn*5* allele within *topA* null but not *topA*$^+$ strains.** Strains were grown in LB medium to $OD_{600}$ of 0.6 at 37°C. Genomic DNA was prepared essentially as described by Nordman *et al.* (2007). Following genomic DNA extraction, samples were digested with XmnI and electrophoresis was performed in 0.8% agarose in 0.5X TBE at 45V for 24 h at room temperature. After electrophoresis, samples were transferred onto a nitrocellulose membrane (Hybond-N GE Healthcare) and hybridized with a $^{32}$P-dCTP-labelled probe obtained by PCR using the primers forward 5′- CATTGGCGGGGGTCATGC-3′ and reverse 5′-CTTGCTCTCCAGCGTCGG-3′ corresponding to the *gidA* gene. The bands were visualised with a Phosphorimager Typhoon 9400 (GE Healthcare). The strains used are: RFM443 (wild-type), RFM443 *kan*$^r$ (wild-type *kan*$^r$: a false positive), RFM475 (*topA gyrB (Ts)*) and VU155 (RFM475 *oriC15::aph*).

Fig. S4


x
gidA
oriC
mioC
x
Xmn1
Xmn1
4057 bp fragment

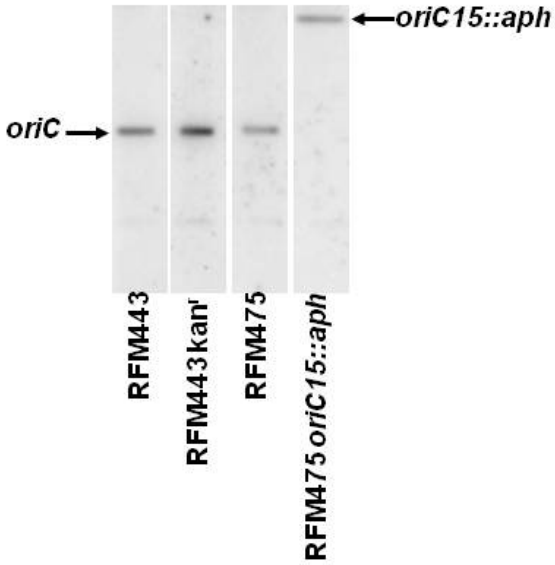

oriC15::aph
oriC
RFM443
RFM443 kan'
RFM475
RFM475 oriC15::aph

Fig. S5

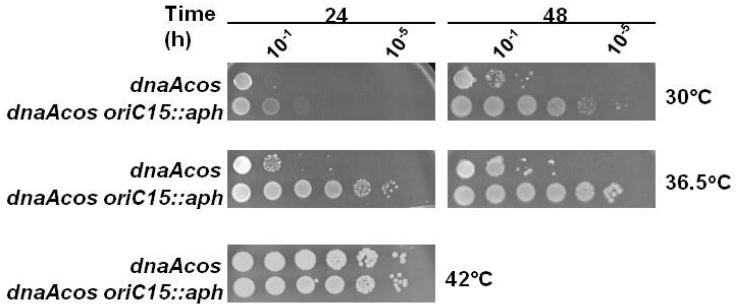
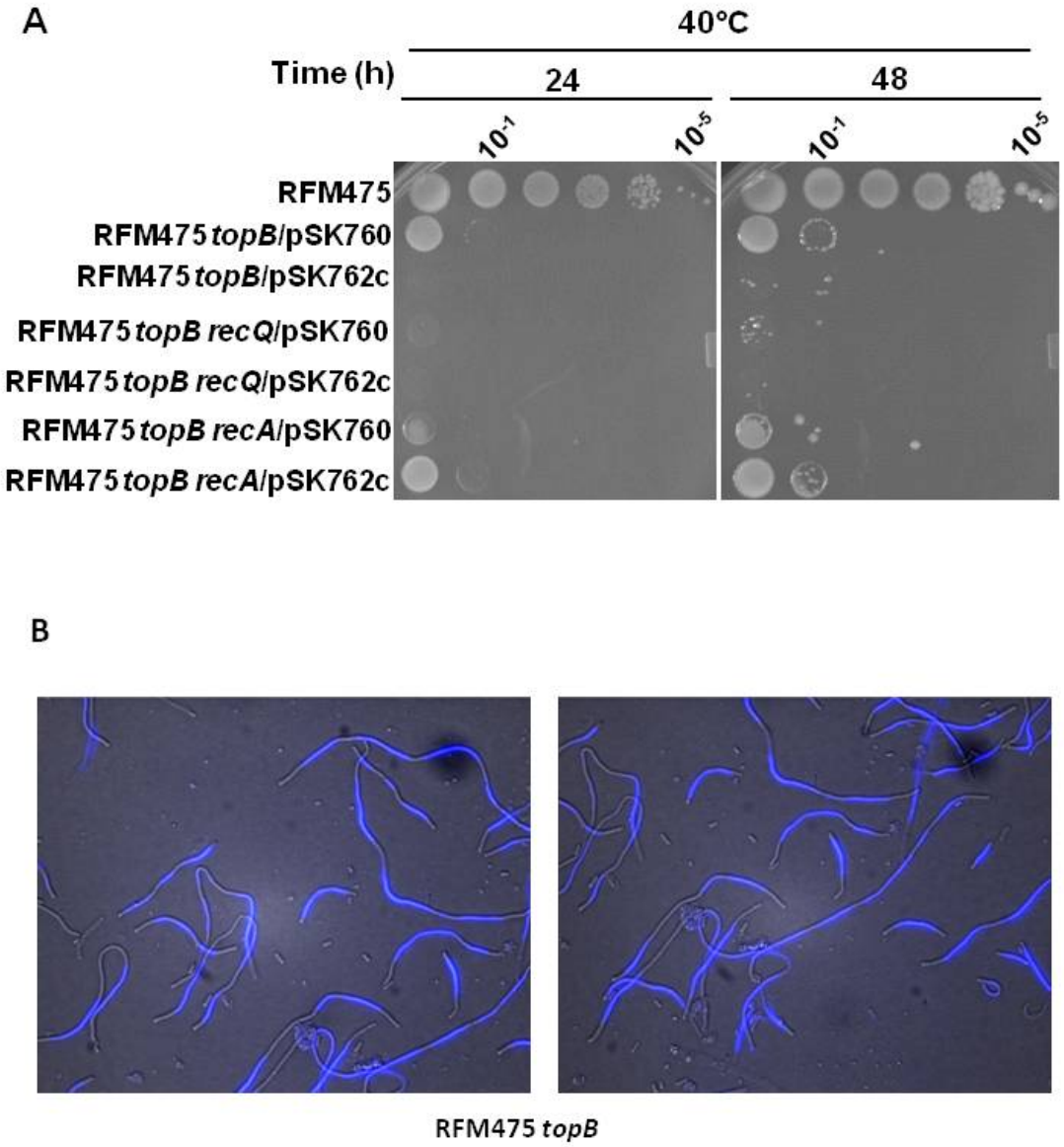


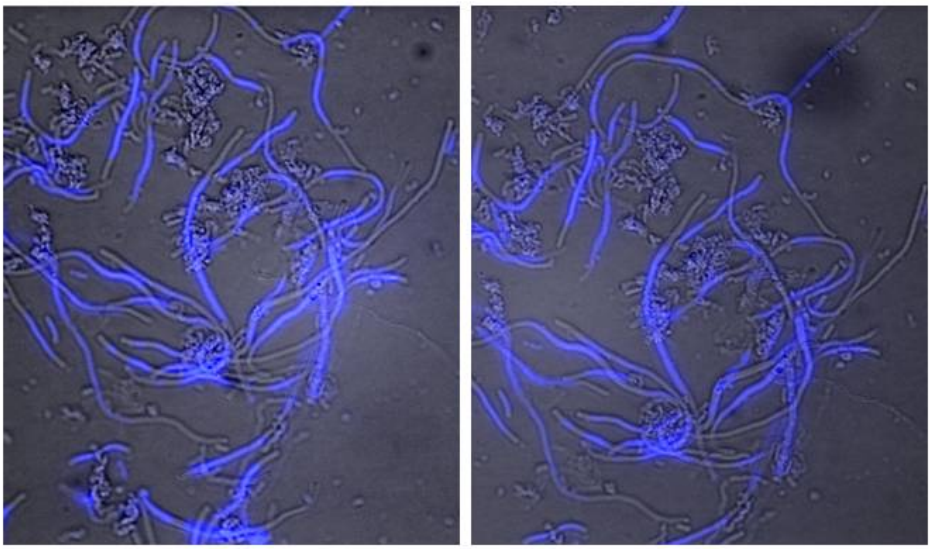

**Figure. S5. The *oriC15::aph* mutation complements the growth defect of a *dnaAcos* mutant at 30°C.** The LB plates were incubated for the indicated time and at 30, 36.5 or 42°C as shown. The strains used were: KA441 (*dnaAcos*) and VU194 (KA441 *oriC15::aph*).

**Figure. S6. Deleting *recA* or *recQ*, or overproducing RNase HI do not complement the growth and chromosome segregation defects of a *topA topB gyrB*(Ts) strain at 40°C.** (a) Cells were spotted on LB plates and incubated at 40°C. The LB plates were photographed after 24 and 48 h of incubation. The strains used are all derivative of RFM475 (Δ*topA, gyrB* (Ts)). They are: VU306 (RFM475 *topB*/pSK760), VU333 (RFM475 *topB*/pSK762c), VU363 (RFM475 *topB recQ*/pSK760), VU365 (RFM475 *topB recQ*/pSK762c), VU375 (RFM475 *topB recA*/pSK760) and VU379 (RFM475 *topB recA*/pSK762c). pSK760 carries the *rnhA* gene for RNase HI overproduction, whereas pSK762C carries a mutated and inactive *rnhA gene*. CT170 (*topA topB gyrB*(Ts)), (b), VU243 (*topA topB gyrB*(Ts) *recA*), (c) and VU205 (*topA topB gyrB*(Ts) *recQ*), (d) cells were prepared for microscopy as described (Usongo *et al*., 2008). Shown are superimposed images of phase contrast and fluorescence pictures of DAPI-stained cells grown at 40°C. (Usongo V, Tanguay C, Nolent F, Bessong JE, Drolet M (2008) Interplay between type 1A topoisomerases and gyrase in chromosome segregation in *Escherichia coli*. Mol Micro 195:1758-1768.).

Fig. S6

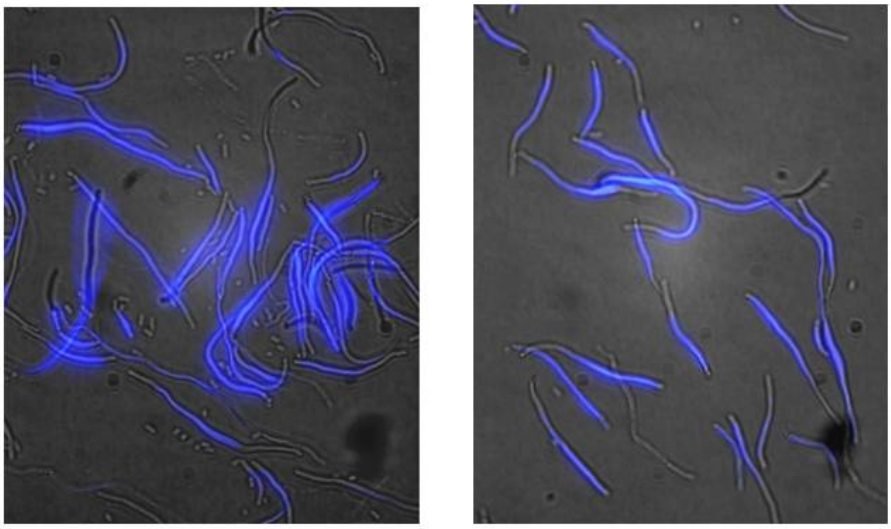

A
40°C
Time (h)
24
48
10-1
10-5
10-1
10-5
RFM475
RFM475 topB/pSK760
RFM475 topB/pSK762c
RFM475 topB recQ/pSK760
RFM475 topB recQ/pSK762c
RFM475 topB recA/pSK760
RFM475 topB recA/pSK762c
B
RFM475 topB

C



RFM475 *topB recA*

D



RFM475 *topB recQ*

**Figure. S7. The growth and chromosome segregation defects of a *topA topB gyrB*(Ts) strain are more severe at 30 than 37°C.** (a) Cells were spotted on LB plates and incubated for 24 h at the indicated temperature. (b) Cells were prepared for microscopy as described (Usongo *et al*., 2008). Shown are superimposed images of phase contrast and fluorescence pictures of DAPI-stained cells grown at 37 or 30°C as indicated. The strains used are all derivative of RFM475 (Δ*topA, gyrB* (Ts)). They are: VU170 (RMF475 *topB*), VU306 (CT170/pSK760) and VU333 (CT170/pSK762c). pSK760 carries the *rnhA* gene for RNase HI overproduction, whereas pSK762C carries a mutated and inactive *rnhA gene*. (Usongo V, Tanguay C, Nolent F, Bessong JE, Drolet M (2008) Interplay between type 1A topoisomerases and gyrase in chromosome segregation in *Escherichia coli*. Mol Micro 195:1758-1768.).

Fig. S7

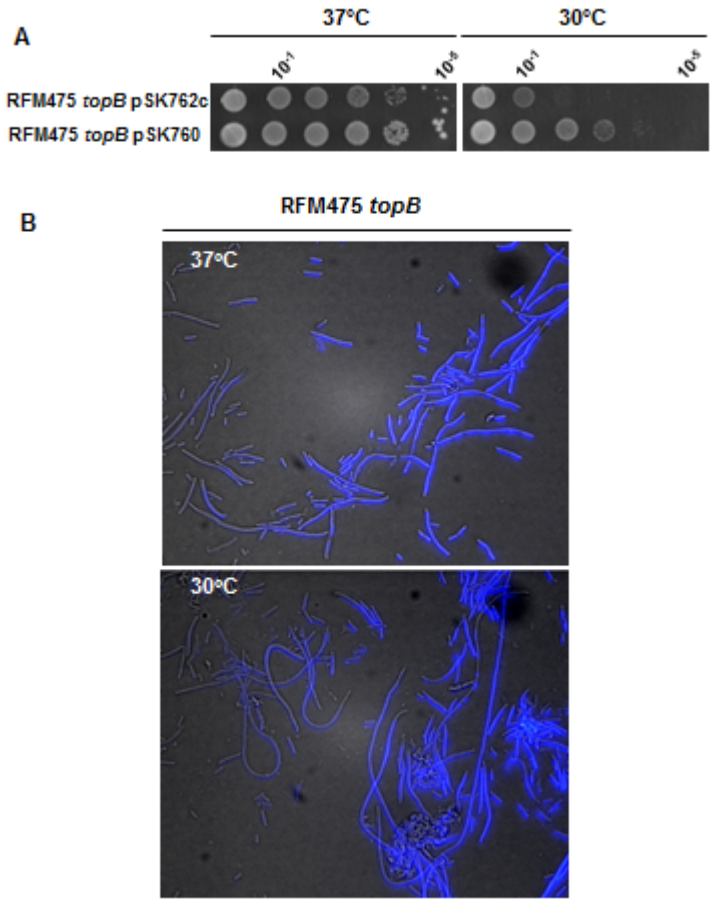

A
37°C
30°C
$10^{-1}$
$10^{-5}$
$10^{-1}$
$10^{-5}$
RFM475 topB pSK762c
RFM475 topB pSK760
B
RFM475 topB
37°C
30°C

**Figure. S8. *aph* insertion sites for three suppressor mutants used in this study.** The *aph* insertion sites for the *dnaT18::aph* (a), *holC2::aph* (b) and *rne59::aph* (c) alleles are shown. For (a) and (b) we show the nucleotide sequence of the regulatory regions for *dnaT* (promoter has been characterized) and *holC* (promoter unknown) where the *aph* cassette was inserted. In (c) we show the amino acids sequence of RNase E. Note that the *aph* cassette is inserted within the protein scaffold region (position 883 for *rne59::aph*) that is used by RNase E to interact with other proteins to form the RNA degradosome (RhlB helicase, Enolase and PNPase) (for details see Mackie (2013)). (Mackie GA (2013) RNase E: at the interface of bacterial RNA processing and decay. (Nat Rev Microbiol 11:45-47.).

Fig. S8

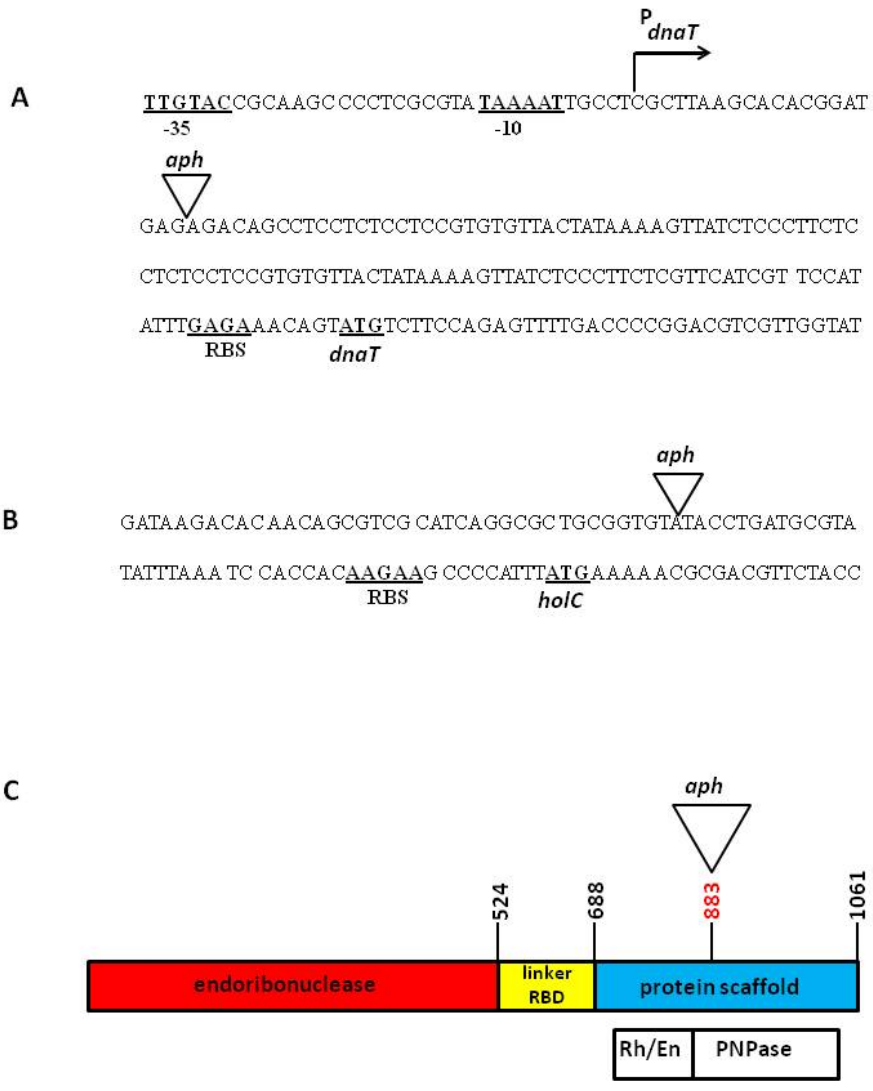

A
P dnaT
TTGTACCGCAAGCCCTCGCGTA TAAAATTGCCTCGCTTAAGCACACGGAT
-35
-10
aph
GAGAGACAGCCTCCTCTCCTCCGTGTGTTACTATAAAAGTTATCTCCCTTCTC
CTCTCCTCCGTGTGTTACTATAAAAGTTATCTCCCTTCTCGTTCATCGT TCCAT
ATTTGAGAAACAGTATGTCTTCCAGAGTTTTGACCCCGGACGTCGTTGGTAT
RBS
dnaT
B
aph
GATAAGACACAACAGCGTCG CATCAGGCGC TGCGGTGTATACCTGATGCGTA
TATTTAAA TC CACCACAAGAAG CCCCATTTATG AAAAACGCGACGTTCTACC
RBS
holC
C
aph
524
688
883
1061
endoribonuclease
linker RBD
protein scaffold
Rh/En
PNPase

Fig. S9

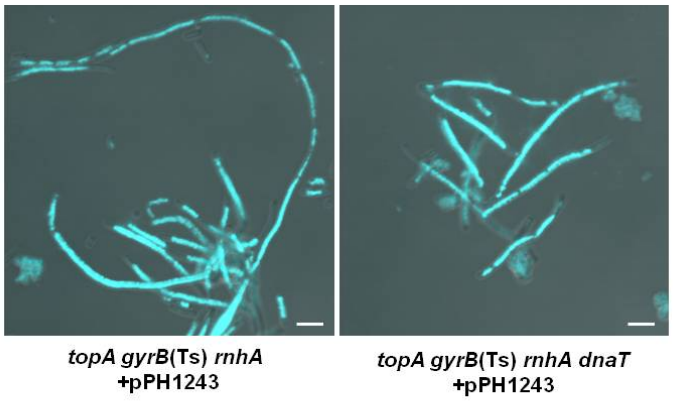



**Figure. S9. The effect of *dnaT18::aph* allele on the chromosome segregation defects of a *topA rnhA gyrB*(Ts) strain**. Superimposed images of DIC and fluorescence pictures of DAPI-stained cells grown at 37ºC in LB without IPTG. Size bars are 5 μm. The strains used are all derivatives of RFM475 (Δ*topA gyrB (*Ts)). They are: VU129 (RFM475 *rnhA*/pPH1243) and VU148 (VU129 *dnaT*). pPH1243 carries the *topB* gene under the control of an IPTG-inducible promoter.

Fig. S10

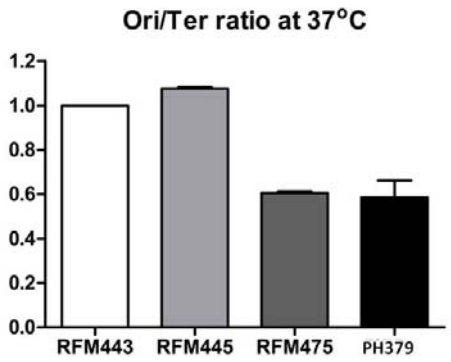



**Figure. S10. The *ori/ter* ratio is similarly reduced in strains lacking either *rnhA* or *topA***. Growth of the strains, genomic DNA extraction and cutting, and Southern blotting were performed as described in the legend to Fig. S4. For the "*ori*" probe, the DNA fragment including the *gidA* gene as described in the legend to Fig. S4 was used. For the "*ter*" probe, a DNA fragment including the *cedA* gene (obtained from PCR with the following primers: 5'-GTTACGCGTATCAGGGGC-3' and 5'-GAGCGACGCCACAGGATG-3') was used. Strains used were: RFM443 (wild-type), RFM445 (*gyrB* (Ts)), RFM475 (*topA gyrB* (Ts)) and PH379 (*rnhA*). The ori and ter bands were visualised and by using a Phosphorimager Typhoon 9400 (GE Healthcare). The "*ori*" and "*ter*" bands were visualised and the signal quantified by using a Phosphorimager Typhoon 9400 and the ImageQuant software (GE Healthcare). Sown here are the results of two independent experiments.

**Fig. S11**

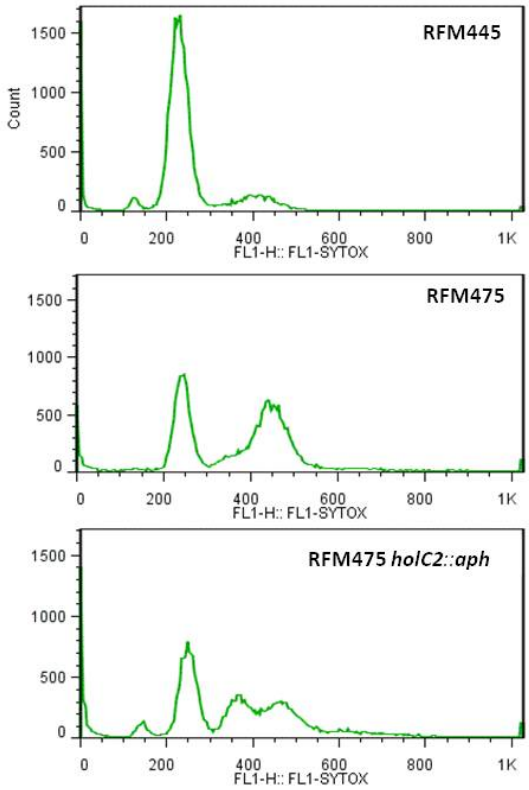



**Figure. S11. Replication initiation asynchrony conferred by the *holC2::aph* mutation.** Rifampicin run-out experiments for flow cytometry analysis were performed as described in Materials and Methods. Cells were grown in M9 minimal medium. The strains used are: RFM445 (*gyrB* (Ts)), RFM475 (*topA gyrB* (Ts)) and VU176 (RFM475 *holC2::aph*).

# CHAPTER 5: Discussion

## 5.1. Supercoiling inhibition in triple *topA rnhA gyrB*(Ts) *mutants*

In the first part of this project, we found that depleting RNase HI activity in a *topA gyrB*(Ts) mutant led to extreme DNA relaxation. This was however surprising because we predicted that the triple *topA rnhA gyrB*(Ts) mutant would die from toxic levels of negative supercoiling based on previous observations whereby overproducing RNase HI was shown to suppress the growth defect of *topA* mutants by relaxing hypernegatively supercoiled DNA (Drolet et al., 1995). DNA from the triple *topA rnhA gyrB*(Ts) mutant was extremely relaxed and we equally found that the cells of triple *topA rnhA gyrB*(Ts) mutants formed long filaments full of DNA reminiscent of chromosome segregation defects (Usongo et al., 2008). Depleting RNase HI activity in triple *topA rnhA gyrB*(Ts) mutants thus triggered a response that leads to the inhibition of gyrase activity (Usongo et al., 2008). This inhibition was reversed when RNase HI expression was induced from a plasmid borne-gene implying that the observable effects were not due to a mutation. This inhibition was unrelated to the SOS response that is expressed when cells lack RNase HI (Kogoma et al., 1993). We confirmed this with our observation that introducing the *lexA3* allele (this allele cannot be cleaved by RecA to enable the expression of SOS control genes) into the triple *topA rnhA gyrB*(Ts) mutant to prevent the induction of genes under the control of the SOS regulon, did not restore supercoiling in the triple *topA rnhA gyrB*(Ts) mutant (Usongo, unpublished results).

To ascertain whether an inhibitor may be produced following the depletion of RNase HI activity, we assayed gyrase activity in crude cell extracts. We found that the ATP-independent relaxation of supercoiled pBR322 DNA was similar in extracts of *topA*$^{-}$ cells irrespective of the presence of the *rnhA* gene, while the ATP-dependent supercoiling reaction was much higher in *topA*$^{-}$ cells carrying the wild-type *rnhA* allele (Usongo, unpublished results). The relaxation observed in extracts of *topA*$^{-}$ cells could have been due either to the relaxation activity of topo IV, or to the ATP-independent relaxation activity of gyrase. To test this, we first constructed norfloxacin-resistant (a *gyrA nal*$^{r}$ allele was used)

derivatives of our *topA* strains. Gyrase and topo IV are both sensitive to norfloxacin however, in this construct, gyrase was rendered resistant by the *nal*$^{r}$ allele so only topo IV was specifically targeted. Relaxation in the absence of ATP was only observed in cell extracts with the *gyrA nal*$^{r}$ allele confirming that the relaxation activity was from gyrase and not topo IV. These results confirm that topo IV is not responsible for the extensive DNA relaxation observed in triple *topA rnhA gyrB*(Ts) mutants, and also confirm that the ATP independent relaxation activity of gyrase was functional. Altogether these results strongly suggest that depleting RNase HI activity triggered a cellular response that inhibited the ATP-dependent activity of gyrase but not its ATP-independent activity.

Further corroborating the fact that the response targeted only the supercoiling activity of gyrase were results from an *in vivo* time course plasmid supercoiling analysis which showed that downshifting *topA*$^{-}$ cells from 37 to 28 $^{o}$C to reactivate gyrase stimulated hypernegative supercoiling in *rnhA*$^{+}$ cells and DNA relaxation in *rnhA*$^{-}$ ones (Usongo, unpublished results). Thus, these results clearly showed that the Ts gyrase was reactivated in both strains, but that only its ATP-independent relaxation activity was functional in *rnhA*$^{-}$ cells. Thus, depleting RNase HI activity triggered a response that specifically inhibited gyrase supercoiling activity. Several models can be advanced to explain why the ATP dependent supercoiling reaction in our triple mutant is defective. Supercoiling inhibition in triple *topA rnhA gyrB*(Ts) mutants could be related to over-replication of the chromosome. Being the only enzyme that introduces negative supercoils in bacteria, gyrase plays a key role in the cell. As previously mentioned, *rnhA* mutants undergo cSDR (Kogoma, 1997) and in addition to replication from *oriC*, this leads to an increase in the overall rate of replication. Excess replication may overwhelm the cellular capacity of the cell as gyrase will be recruited to several sites on the chromosome thereby lowering its efficacy. The depletion of RNase HI activity may thus trigger a cellular response that inhibits gyrase activity as a means to protect the chromosome from excess replication which can be pathologic for the cell.

Supercoiling inhibition in our triple mutant could be attributed to the endogenous gyrase inhibitors such as MurI and GyrI earlier described. GyrI (encoded by s*mbC*) is under

the control of the SOS regulon (Chatterji & Nagaraja, 2002). GyrI prevents the interaction between gyrase and DNA. As shown in chapter two, deleting *smbC* had no effect in our triple mutant. Whereas the *topA gyyrB*(Ts) mutant became hypernegatively supercoiled following a downshift to 28ºC, the triple *topA rnhA gyrB*(Ts) mutant remained relaxed implying that GyrI was not responsible for the extensive DNA relaxation observed in the triple *topA rnhA gyrB*(Ts) mutant. MurI, another gyrase inhibitor, could also be responsible for supercoiling inhibition in the triple *topA rnhA gyrB*(Ts) mutant. MurI inhibits gyrase only in the presence of the peptidoglycan precursor UDP-N-acetyl muramyl-l-alanine (UDP-MurNAc-L-Ala) the racemase activator (Ashiuchi et al., 2002). In *E. coli*, it has been shown that the absence of MurI leads to cell filamentation (Doublet et al., 1992) while overexpressing MurI lead to a reduction in the negative supercoiling level of the chromosome, cell filamentation and segregation defects (Baliko & Venetianer, 1993). MurI inhibits gyrase by preventing it from gaining access to the DNA substrate (Sengupta, Shah, & Nagaraja, 2006). Northern blot experiments from RNA extracted from the triple *topA rnhA gyrB*(Ts) mutant showed that MurI was not overexpressed in this mutant implying that MurI was not responsible for supercoiling inhibition in the triple *topA rnhA gyrB*(Ts) mutant (Usongo, unpublished results).

Supercoiling inhibition in the triple *topA rnhA gyrB*(Ts) mutant can also be attributed to the abundant nucleoid associated proteins such as HU and Fis that interact with DNA (Travers & Muskhelishvili, 2005ab). HU stimulates gyrase activity *in vitro* (Marians, 1987). HU absence leads to slow growth and partial relaxation of the chromosome which is corrected by a mutation in *gyrB* (Malik et al., 1996). Northern blot experiments from RNA extracted from the triple *topA rnhA gyrB*(Ts) mutant clearly showed that there was no difference in HU expression levels in *topA gyrB*(Ts) and *topA rnhA gyrB*(Ts) implying that supercoiling inhibition in the triple *topA rnhA gyrB*(Ts) mutant was unrelated to HU expression (Usongo, unpublished results). Fis controls the transcription of genes coding for DNA topoisomerases (Cameron et al., 2011) and directly affects the supercoiling activity of gyrase (Cameron et al., 2011). Western blot experiments on protein extracts from a wild type, *topA gyrB*(Ts), and the triple *topA rnhA gyrB*(Ts) mutant clearly showed that there was no change in Fis expression levels (Usongo, unpublished results) implying that

supercoiling inhibition in the triple *topA rnhA gyrB*(Ts) mutant was unrelated to Fis expression.

In the cell, ATP is produced via the Krebs cycle and glycolysis. It is possible that in the triple *topA rnhA gyrB*(Ts) mutant, the depletion of RNase HI activity may affect the metabolic pathway that synthesizes ATP in the cell, limiting its availability. This may explain why supercoiling was observed only in *topA*$^{-}$ *gyrB*(Ts) both *in vivo* and in crude cell extracts. When RNase HI is depleted, the pathway leading to the production of ATP may be affected, leading to a reduction in ATP production. In this regard, supercoiling inhibition in the triple *topA rnhA gyrB*(Ts) mutant may be viewed as an indirect effect of RNase HI depletion which affects ATP production. Because ATP is the energy currency of the cell, competition for it will limit its availability. Reducing processes that consume ATP such as DNA replication will make more ATP available for the supercoiling reaction of gyrase. This may explain why supercoiling was restored in suppressors that generally affected DNA replication.

Alternatively, it can be argued that depleting RNase HI activity may induce the synthesis of a protein that consumes ATP and this may explain the supercoiling inhibition observed when wild type extracts were mixed with extracts of the triple *topA rnhA gyrB*(Ts) mutant. In this experiment, cells of a wild type strain and those of the triple *topA rnhA gyrB*(Ts) mutant were grown at 37$^{o}$C. In addition, cells of the triple *topA rnhA gyrB*(Ts) mutant were downshifted to 28$^{o}$C for 60 minutes to reactivate gyrase. Crude cell extracts prepared from the wild type strain were mixed with extracts from the triple *topA rnhA gyrB*(Ts) mutant and these extracts were assayed for the ATP dependent supercoiling reaction of gyrase based on their ability to supercoil relaxed pBR3322 DNA in the presence of ATP. Wild type extracts were not inhibited by extracts from a *topA gyrB*(Ts) mutant. To find out whether a protein present in the extract was consuming ATP, we did an ATP time course experiment. We found that increasing concentrations of ATP stimulated gyrase supercoiling activity to the same extent in the crude cell extracts of the triple *topA rnhA gyrB*(Ts) mutant and in extracts of *topA*$^{-}$ *gyrB*(Ts) (Usongo, unpublished results). These results clearly indicated that the supercoiling inhibition in the triple *topA rnhA gyrB*(Ts) mutant was not due to the presence of an intracellular factor that consumed ATP

at the expense of gyrase for if this was the case, we should not have seen an increase in gyrase activity in the extracts of of *topA*$^{-}$ *gyrB*(Ts).

Other observations suggest that the inhibitor of gyrase in the triple *topA rnhA gyrB*(Ts) mutant is protein in nature. We observe that when extracts of the triple *topA rnhA gyrB*(Ts) mutant were heated prior to *in vitro* supercoiling assays, gyrase inhibition was abolished. Supercoiling was restored in extracts from a wild-type strain when mixed with heated extracts of the triple *topA rnhA gyrB*(Ts) mutant as opposed to unheated extracts (Usongo, unpublished results). It is possible that supercoiling inhibition in the triple *topA rnhA gyrB*(Ts) mutant could be due to a combination of factors. The depletion of RNase HI activity as much as it might trigger the synthesis of a gyrase inhibitor as our data indicate, it may also affect the metabolic pathway that synthesizes ATP in the cell. Also, excess replication in the triple *topA rnhA gyrB*(Ts) mutant may warrant that many gyrase molecules be recruited leaving only few molecules available for supercoiling.

### 5.2. Suppressors of *topA rnhA gyrB*(Ts) mutants

To better understand the mechanism(s) of supercoiling inhibition in the triple *topA rnhA gyrB*(Ts) mutants and potentially identify a gyrase inhibitor, we used a genetic approach to identify suppressors of this mutant taking advantage of its inability to grow at 40$^{o}$C. The growth defect of *gyrB*(Ts) single mutants at high temperatures has been attributed to the inability of these mutants to initiate replication at *oriC* as a result of a decrease in supercoiling. This growth defect can be corrected by deleting *topA* which leads to an increase in chromosomal supercoiling (Usongo et al., 2013). To identify suppressors of the triple *topA rnhA gyrB*(Ts) mutant, the highly efficient transposon mutagenesis system developed by Metcalf and co-workers (Larsen et al., 2002) was used for the suppressors search. In this construct, the transposon delivery vector pRL27 has a hyperactive Tn*5* transposase which is 1000-fold more active than the wild type transposase. Moreover, the gene encoding the transposase is located outside of the transposon so that after transposition; the transposase is lost, resulting in stable transposon insertions. This vector is incapable of replicating in a host that lack *pir* as was the case in the triple mutant.

The transposon has a gene encoding for $Km^r$, as a selectable marker. This system has been successfully used in *E. coli* to map suppressors (Bradshaw & Kuzminov, 2003; Budke & Kuzminov, 2010).

Following this approach, we identified three major categories of suppressors. Most of these suppressors corrected the supercoiling defect as well as the growth and chromosome segregation defects of the triple *topA rnhA gyrB*(Ts) mutant. The first category of suppressors was insertions in genes/loci implicated in DNA replication and among these category were insertions in *holC* involved in clamp loading and processivity (Marceau et al., 2011), *dnaT* implicated in replisome loading in SDR and cSDR and *oriC*. However, the majority of the suppressors in this category were insertions in genes implicated in nucleotide metabolism. The second category of suppressors was insertions in *rne* encoding the endoribonuclease RNase E and *rnr* encoding the ribonuclease RNase R. Insertions in *rne* and *rnr* restored supercoiling and corrected the growth and segregation defect of *topA*$^-$ *rnhA*$^-$ *gyrB*(Ts) mutant likely by preventing the formation of R-loops which can be used for cSDR (Kogoma, 1997). The third category of suppressors was insertions in diverse genes with some of unknown functions. The majority of the suppressors in this category were insertions in genes involved in the synthesis of type 1 fimbriae with most insertions in genes linked to swarming. In this category, we also obtained an insertion in *pta* encoding Pta (phosphate acetyl transferase) which catalyzes the reversible conversion between acetyl-CoA and acetyl phosphate and *aceE* (component of the pyruvate dehydrogenase multi-enzyme complex) that converts pyruvate to acetyl-CoA. These mutants make less acetyl-CoA. Acetyl-CoA is the high energy intermediate that sits at the crossroad of central metabolism.

### 5.2.1. Correction by transposon insertions in nucleotide biosynthetic genes

Insertions in genes involved in nucleotide biosynthesis may affect the deoxynucleoside triphosphate (dNTP) pool. The link between dNTP synthesis and DNA replication has long been established by studies which showed that dNTP synthesis is coordinated with DNA replication (Mathews & Sinha, 1982; Pato, 1979; veer Reddy &

Pardee, 1982). The dNTP pool is highly regulated and it has been found that its level in the cell is about 1% of the amount required for a round of DNA replication (Kornberg & Baker, 1992). One important regulator of dNTP pool levels in the cell is ribonucleoside diphosphate reductase (rNDP reductase) encoded by the *nrdAB* genes. The precursors for DNA synthesis, deoxyribonucleotides are produced by direct reduction of the corresponding ribonucleotides catalysed by rNDP reductase (Thelander & Reichard, 1979). DnaA protein in its ATP-bound form represses the transcription of *nrdAB* genes (Messer, 2002). After initiation of DNA replication, DnaA-ATP is rapidly converted to DnaA-ADP and this result in the derepression of *nrdAB* transcription. This derepression allows the correct supply of dNTPs for replication (Gon et al., 2006). The correction of DNA supercoiling and chromosome segregation defects in the triple *topA rnhA gyrB*(Ts) mutant by suppressors in this category could be explained by a reduction in DNA replication as a result of a decrease in the dNTP pool. A reduction in the dNTP pool will reduce replication from *oriC* as well as *oriC* independent replication (cSDR). An overall reduction in replication will imply that less gyrase will be consumed and more will be available for supercoiling.

### 5.2.2. Correction by transposon insertions in genes implicated in fimbriae formation

Supercoiling was restored both *in vivo* and in crude cell extracts in mutants with insertion in genes involved in fimbriae synthesis. Cells for supercoiling assays *in vivo* and in crude cell extracts were grown at 37$^{o}$C, and downshifted to 28$^{o}$C for 60 minutes to reactivate gyrase. Supercoiling restoration can be explained by several reasons. A study in *E. coli* identifying swarming related genes also picked up most of the genes identified in our screen, and in this study, it was reported that both swimming and swarming use a lot of cellular energy (Inoue et al., 2007). Inactivating genes involved in swarming/swimming may make more ATP available for gyrase to power supercoiling. This can be explained in a model which posits gyrase acting as a metabolic sensor that bridges environmental conditions to physiological adaptation. In this context, gyrase senses the energy status of the cell through the ATP/ADP ratio and its ATPase activity. When the ratio is low, especially under unfavorable growth conditions, gyrase inhibition leads to fimbriae

synthesis which consumes the remaining ATP available. This in turn, causes extensive DNA relaxation and growth inhibition. In support of this model, decreasing gyrase activity following novobiocin treatment was shown to stimulate fimbriae synthesis (Muller et al., 2009). Fimbriae formation can then trigger a transition from a planktonic to an attached life style culminating in the formation of biofilms. When the sensor that triggers the transition is dysfunctional, which may be the case in the *topA rnhA gyrB*(Ts) mutant, the transition mode may be triggered while the cells still have enough energy to grow; implying that the cell cycle, including replication and chromosome segregation are still on, so the extensive gyrase inhibition now will lead to severe chromosome segregation defects as seen in the triple *topA rnhA gyrB*(Ts) mutant. Inhibiting the formation of fimbriae with suppressor mutations in genes involved in fimbriae synthesis, will thus make a great amount of cellular ATP at the disposal of gyrase, thus correcting the supercoiling, chromosome segregation and growth defects.

Another possibility is that when the ATP/ADP ratio is low, supercoiling inhibition results in DNA relaxation which then induces fimbriae synthesis plus a gyrase inhibitor that may be a fimbriae subunit.

### 5.2.3. Correction by transposon insertions in genes implicated in central metabolism

The correction of growth and chromosome segregation defects as well as supercoiling in triple *topA rnhA gyrB*(Ts) mutant by insertion in genes that lead to a reduction in acetyl-CoA production could be explained by two models: one involving protein acetylation and the other linking acetyl-CoA to central metabolism. CheY, a protein responsible for bacterial chemotaxis controlling flagella movement has been shown to be activated by acetylation in addition to phosphorylation. Two mechanisms of CheY acetylation have been identified: acetylation by acetyl CoA synthetase (Acs) with acetate or acetyl coenzyme A (AcCoA) as an acetyl donor (Barak et al., 2004; Barak et al., 1992) and autoacetylation (Barak et al., 2006) with AcCoA as the acetyl donor. Studies have shown that both phosphorylation and acetylation determine the ability of CheY to bind to its target protein and provide two levels of regulation, fast and slow respectively. Environmental signals such as chemotactic stimuli trigger the fast level while the slow level is modulated

by the metabolic state of the cell (Liarzi et al., 2010). CheY generates clockwise rotation and thus favors swarming, an energy expensive activity that consumes a lot of ATP. Therefore, inactivating CheY acetylation by a reduction in acetyl-CoA pool reduces energy consumption by flagella movement and more ATP is made available for supercoiling. This may explain the correction of the triple *topA rnhA gyrB*(Ts) mutant by insertions in *pta* and *aceE* that both reduce the acetyl-CoA pool.

Supercoiling restoration of the triple *topA rnhA gyrB*(Ts) mutant with insertions in genes that reduce acetyl-CoA production can also be explained by the link between acetyl-CoA and central metabolism (Wolfe, 2005). A link between central carbon metabolism and DNA replication has been established following studies conducted in *E. coli* as well as in *Bacillus subtilis*. In *B. subtilis*, suppressors of temperature sensitive replication mutants were detected only in genes encoding enzymes that act at the late stages of glycolysis and gluconeogenesis (Janniere et al., 2007). In *E. coli*, the correlation between central metabolism and DNA replication was also demonstrated with the findings that the temperature sensitivity of mutants coding for replication proteins could be suppressed by mutations coding for enzymes involved in central carbon metabolism (Maciag et al., 2011). It was also found in another *E. coli* study that the cell division defects of various replication mutants could be corrected by mutations in genes involved in central carbon metabolism (Maciag-Dorszynska et al., 2012). In both *E. coli* studies, one of the suppressors was *pta* which we picked up in our suppressor screen. These studies clearly established a link between central carbon metabolism and DNA replication initiation and elongation. Therefore, the correction of the phenotypes of the triple *topA rnhA gyrB*(Ts) mutant by insertion mutations that reduce acetyl-CoA levels could be linked to ATP availability. Indeed, since acetyl-CoA sits at the cross road of central metabolism, reduction in its level may lead to an overall decrease in metabolism implying that processes such as DNA replication will be slowed down. An overall reduction in DNA replication will increase the ATP pool in cells and thus makes more ATP available for gyrase.

## 5.3 type IA topos and genome maintenance

In the second part of my project, the role of type IA DNA topoisomerases in DNA metabolism in *E. coli* was studied using a genetic approach to reveal the *in vivo* functions of these topoisomerases. It is not an accident of nature that at least one type IA DNA topoisomerase is required in all living organisms spanning from *E. coli* to humans. Several studies have pointed out that one important function of type IA DNA topoisomerases is in the resolution of DNA structures that are formed during recombination and clues to this, first emerged with studies in yeast. However, we found that the role of type IA DNA topoisomerases is not only limited to the resolution of homologous recombination intermediates. They equally play a role in DNA replication initiation and contribute to the overall maintenance of a stable genome.

### 5.3.1. Type IA topoisomerases and genome maintenance in *E. coli*

We have demonstrated interplay between gyrase and type IA DNA topoisomerases in chromosome segregation. We found that both the growth defect and Par- phenotypes of *gyrB*(Ts) cells at the nonpermissive temperature were corrected by deleting *topA* only in the presence of *topB* (Usongo et al., 2013). Unlike topoisomerases of types IB and II which have well defined and characterized roles in DNA synthesis, type IA topoisomerases have mostly been associated with DNA recombination irrespective of the fact that their participation in replication has been clearly demonstrated *in vitro* through reconstituted systems (Hiasa & Marians, 1994a, 1996). Their role in recombination has been illustrated *in vivo* by the appearance of long filaments with unsegregated nuclei in cells genetically depleted of their activity (Zhu et al., 2001., Usongo, unpublished results). Moreover, the work of Digate and coworkers (Zhu et al., 2001) was originally interpreted as indicating the involvement of type 1A topos in recombination because their phenotype was corrected by *recA*$^-$; we have shown the same correction by *recA*$^-$ in double *topA topB* mutants. We have further investigated this phenotype and rather found that the involvement of RecA is in the frame of cSDR, thus showing that type 1A topos act together in replication (Usongo, unpublished results). Moreover, our observation that deleting *topA* corrects the phenotypes

of *gyrB*(Ts) cells at the nonpermissive temperature only when *topB* is present clearly establishes an *in vivo* role for type IA topos in DNA replication that is unrelated to the resolution of recombination intermediates. This role is supported by our observations that deleting *recA,* a compensatory mechanism that significantly corrected the growth and segregation defects of a *topA topB gyrB*(Ts) mutant at lower temperatures, had no effect at high temperatures (Usongo, unpublished results). *topA topB* null mutants have been shown to suffer from severe RecA- dependent segregation defects (Zhu et al., 2001) so it will be reasonable to argue that the growth and segregation defects of a *topA topB gyrB*(Ts) mutant at the nonpermissive temperature is RecA-dependent which was however not the case. More importantly, we found that overproducing topo III at a very high level corrected the Par- phenotype of the *gyrB*(Ts) strain at the nonpermissive temperature (Usongo et al., 2013). As mentioned earlier, the growth and chromosome segregation defects of triple *topA rnhA gyrB*(Ts) mutant was corrected by overproducing topo III. We found that in the triple *topA rnhA gyrB*(Ts) mutant substituting the *gyrB*(Ts) allele by a $gyrB^{+}$ one bypassed the need for topo III overproduction for growth and chromosome segregation in the triple mutant (Usongo et al., 2013). This point to an interplay between type IA topos and gyrase and suggest that at nonpermissive temperatures when gyrase is ineffective, type IA topos play a role in chromosome segregation.

We equally found that the *oriC15::aph* insertion mutation significantly corrected both the growth and chromosome segregation defects of the *topA* mutant at low temperatures. This result clearly indicates that topo I plays an important role in regulating replication initiation at *oriC.* Other compensatory mechanisms that corrected the segregation defects of *topA* mutants included deleting *recQ, recA,* and overproducing topo III. Altogether, these findings point to a role of topo I in resolving homologous recombination intermediates. In fact, topo I is present in all bacteria while topo III is present only in a few of them (Forterre et al., 2007). This may suggest that topo I can normally perform all the functions attributed to a type IA topoisomerase in bacteria. Overproducing topo III also corrected the growth and segregation defects of the triple *topA rnhA gyrB*(Ts) mutant (Usongo et al., 2008). In this case topo III may substitute in part for the role of the more abundant protein topo I in R-loop prevention. In *topA rnhA gyrB*(Ts) cells excess replication is due to both R-loop

primed cSDR and over-initiation from *oriC*. Interestingly, studies in our laboratory have shown that topo III can inhibit R-loop formation both *in vivo* and *in vitro* (Broccoli et al., 2000).

That deleting *recQ* improved the growth of cells lacking both topo III and IV activity (Lopez et al., 2005; Perez-Cheeks et al., 2012), may suggest that topo III, like topo I, can also act with RecQ to resolve recombination intermediates. This is in agreement with our finding that overproducing topo III can substitute for the absence of topo I (Usongo, chapter 4). Thus, by deleting *recQ*, topo III will be freed from RecQ and more of it will be available to help topo IV in removing precatenanes during replication.

### 5.3.2. Type IA topoisomerases and genome maintenance in eukaryotes

Phenotypes of genome instability are also manifested in eukaryotes in the absence of type IA topos. In the budding yeast *S. cerevisiae*, type IA topo was the first enzyme of this family to be identified in eukaryotic cells (Wallis et al., 1989) and since it was the third topo to be identified in this organism, it was named Top3. This topo was discovered following the isolation of a mutation in *top3* that stimulated hyper-recombination between repeated sequences (Wallis et al., 1989). Phenotypes of *top3* mutants include slow growth. In addition, these mutants are also sensitive to DNA damaging agents and are equally defective in forming viable spores during sporulation (Chakraverty et al., 2001; Frei & Gasser, 2000; Mullen et al., 2000; Saffi et al., 2000; Yamagata et al., 1998). The slow-growth and sporulation defect of t*op3* mutants are suppressed by a mutation in *SGS1* (SGS stands for slow growth suppression) encoding the RecQ homolog (Watt et al., 1995; Bennett et al., 1998) of *S. cerevisiae* or by overproducing *E. coli* topo I (Gangloff et al., 1994; Wallis et al., 1989). In fact suppression with the SGS1 mutation bodes well with the observation that Top3 physically and functionally interacts with Sgs1 (Bennett et al., 2000; Gangloff et al., 1994; Goodwin et al., 1999).

The slow growth and sporulation deficiency of *top3* mutants are also suppressed by deleting *Rad51*, encoding the RecA homolog of *S. cerevisiae* (Oakley et al.,2002; Shor et al., 2002). These data suggest that Sgs1 processed recombination intermediates generating structures that can only be resolved by a type IA topo such as Top3 or *E. coli* topo I. A

mutation in *SGS1* abolishes this pathway and prevents the accumulation of toxic recombination intermediates that can only be resolved by Top3. Overproducing *E. coli* topo I suppressed these phenotypes since it can substitute for the absence of Top3 in the processing of recombination intermediates generated by SGS1. This bodes well with our observation that the growth and segregation defects in cells lacking topo I activity were corrected by deleting *recQ, recA,* or by overproducing topo III. Our data suggest that in *E. coli*, topo I acts with RecQ to resolve RecA-dependent recombination intermediates that will otherwise lead to chromosome segregation defects. Our data also suggest that type IA topos act in this recombination pathway downstream of RecA. We found that *recA* and *recQ* deletions were epistatic to *topB* overexpression in correcting the growth and segregation defects of *topA gyrB*(Ts) (chapter 4).

### 5.3.3. Type IA DNA topoisomerases and genome maintenance in higher eukaryotes

The absence of type IA topos in higher eukaryotes also affects the stability of the genome. Vertebrates have two type 1A DNA topoisomerases Top3α and Top3β. Studies in mice have shown that embryos in which both copies of Top3α are deleted die shortly after implantation (Li & Wang, 1998). Mice lacking Top3β have a shorter life span compared to mice with the wild type gene (Kwan & Wang, 2001). They display a decrease in fertility as they age and also develop autoimmunity (Kwan et al., 2007; Kwan et al., 2003; Kwan & Wang, 2001). The development of autoimmunity in mice lacking Top3β is unrelated to problems with B and T lymphocytes but is related to an increase rate of apoptosis due to chromosomal damage (Kwan et al., 2007). The lack of Top3β in cells also affects response to DNA damage. p53 plays an important role in mediating the $G_1$/S checkpoint and in the absence of Top3β, this pathway is not activated following the induction of DNA damage (Mohanty et al., 2008). Zebra fish lacking Top3α die at about ten days after they have been fertilized and they also show a distinct lack of differentiating thymocytes and this defect is corrected by suppressing p53, suggesting that in the absence of Top3α, the DNA damage pathway is activated (Monnich et al., 2010). Top3α is also involved in the embryonic

development of *Drosophila*. When Top3α is absent, embryonic development is arrested at an early stage (Plank et al., 2005).

Chromosome painting of Top3β mutants has also revealed that these mutants exhibit an increase in aneuploidy, hyperploidy and apoptosis in germ line cells (Kwan et al., 2003). These findings were attributed to the ability of eukaryotic type IA DNA topos to resolve meiotic double Holliday junctions. Interestingly, it has been shown that Top3α interacts with BLM the RecQ homolog in humans and this interaction is important for proper chromosome segregation (Chan et al., 2007). BLM is required for the localization of Top3α on anaphase bridge structures (thin strands of DNA stretching between separating chromosomes). These bridges formed at high frequency between segregating sister chromosomes in normal human cells undergoing mitosis. The observation that these bridges were decorated with BLM led to their identification (Chan et al., 2007). In the absence of BLM, the frequency of these bridges increases leading to segregation defects (Chan et al., 2007). In fact it has been shown that the activity of BLM at these bridges limits the lethality associated with Rad51 (encoding the RecA homolog in human cells) down regulation in human cells (Lahkim et al., 2010). Interestingly, our data also reveal that in *E. coli*, RecQ processed recombination intermediates are resolved by a type IA topo and this will explain why the growth and segregation defects of topo I were corrected by deleting *recQ, recA* or by overproducing topo III. Deleting *recA* eliminates this pathway while deleting *recQ* eliminates the RecQ and topo I pathway in the processing of RecA generated recombination intermediates. In addition, it has been shown that in human cells, BLM acts downstream from RAD51 to resolve RAD51-mediated Holliday junctions (Lahkim et al., 2010). Interestingly, we also found an epistatic interaction between RecA and RecQ (the *topA gyrB*(Ts) *recA recQ* mutant had the viability of the *topA gyrB*(Ts) *recA* mutant) in *E. coli* (Usongo, unpublished results). *recQ* and *recA* complement the growth defect of a *topA gyrB*(Ts) strain (chapter 4). We observed that *recQ* complemented the growth defect of a *topA gyrB*(Ts) strain better than *recA* indicating that in *topA gyrB*(Ts) *recA recQ,* RecQ had no effect on viability outside the RecA-dependent processes confirming that RecQ acts downstream of RecA. These observations clearly indicate that

the molecular mechanisms employed in the maintenance of genome stability are conserved among type IA topos.

**5.6. Type IA DNA topoisomerases and the maintenance of a stable mitochondrial genome.**

Type IA topos plays a role in the maintenance of a stable mitochondrial genome in humans as well as other eukaryotes. In mammalian cells, mitochondrial DNA (mtDNA) is the only genetic material that is located outside of the nucleus. mtDNA molecules are close circular and topologically constrained (Moraes, 2001). Type IB toposimerase have been shown to ensure mtDNA replication (Kosovsky & Soslau, 1991; Topcu & Castora, 1995; Tua et al., 1997). A human mitochondrial topo I gene Top1mt encoding a mitochondrial-targeting type IB DNA topoisomerase and homologous to nuclear Top1 has also been identified and shown to possess a mitochondrial localization signal (Zhang et al., 2001). Though it has been shown that the mitochondria in human cells harbor a type IB topoisomerase (Top1mt), knock-out studies in mice have shown that it is not essential (Zhang et al., 2007) suggesting that its role may be substituted by another topoisomerase. Interestingly it has been shown that human Top3α (hTop3α) localizes both in the nucleus and mitochondria (Wang et al., 2002) suggesting that it can substitute for Top1mt. This implies that Top3α can play a major role in mitochondrial genome maintenance. In fact studies have shown that replication of both strands of the close circular genome of the mitochondria is not synchronous (Brown et al., 2005) implying that single strand gaps may persist in the DNA allowing Top3α to gain access to help terminate replication and ensure faithful chromosome segregation. We found that the growth defect and Par- phenotypes of a *gyrB*(Ts) mutant at the nonpermissive temperature were corrected by deleting *topA* only when topo III was present and overproducing topo IV the major cellular decatenase did not substitute for *topB*. More importantly, overproducing topo III at a very high level was shown to suppress the Par- phenotype (Usongo et al., 2013). All these data point to the essential role type IA topos play to ensure the stability of the genome. The maintenance of a stable genome in mitochondria is of great importance judging from the fact that mitochondria produce most of the cellular energy and also defects in mitochondria lead to a

variety of human diseases such as myopathies, diabetes, neurodegeneracies, progeria (early aging) and cancers (Greaves & Taylor, 2006; Schapira, 2006).

The maintenance of stable genome by type IA topos is not only limited to the human mitochondria. Trypanosomatids protozoa which cause important animal and human diseases and include protozoans such as the African trypanosome *Trypanosoma brucei* and *Leishmania* spp also have a complex mitochondrial DNA called kinetoplast (or kDNA) (Shapiro & Englund, 1995). kDNA is a complex network of topologically interlocked DNA circles. In the mitochondrion of each cell, this network of kDNA is condensed into a disc-shaped structure and this network is made up of two circles termed the maxi and mini circles with the former present in few dozen copies and the later in several thousand copies (Shapiro & Englund, 1995). These networks are inherited by daughter cells through replication. Studies have shown that minicircles do not replicate while attached to the network. They replicate via theta structures intermediates as free circles following their release from the network by a type II topoisomerase (Liu et al., 2005) which could be the mitochondrial enzyme Top2mt (Kulikowicz & Shapiro, 2006). Once replication is completed, progeny minicircles are re-attached to the network (Liu et al., 2005). Reattachment could be achieved by a type II topoisomerase such as Top2mt, however, recent studies in *T. brucei* have revealed that this reattachment is performed by a type IA topoisomerase, Top1mt (Scocca & Shapiro, 2008). In this study, it was observed that silencing the expression of Top1mt by RNAi leads to a striking accumulation of kDNA late theta structure intermediates, culminating in the loss of kDNA networks and a halt in cell growth. This study thus provides solid evidence that type IA topoisomerases are absolutely required in the resolution of late theta replication intermediates. The presence of nicks or gaps in the DNA network of kDNA clearly justifies the action of type IA topoisomerase since they are capable of attaching nicked or gaped ring as opposed to intact rings. In kDNA replication, the conversion of replicated rings into the covalently closed rings only occurs after all the minicircles in the network have been replicated, implying that the kinetoplast replication system may use the nicks or gaps to identify a minicircle that has completed replication. This may serve as a cell cycle control mechanism to ensure that each minicircle is replicated once per generation.

In *Drosophila* producing a Top3α that is localized only in the nucleus following a mutation of the start codon upstream of the mitochondrial localization signal also show phenotypes of genome instability (Wu, Feng, & Hsieh, 2010). In this study, mutant flies were shown to have a low mtDNA copy number as well as low ATP content, suggestive of a problem in DNA replication and underperforming mitochondria respectively. It was also observed that female flies were completely sterile while for male flies, a fraction was completely sterile and the other fraction manifested gradual loss in sterility. Progressive germ line stem cell loss was responsible for male sterility. This implies that Top3α, through its mitochondrial function, plays an important role in germ line stem cell maintenance. All these observations point to the important role that type IA topos play in the maintenance of genome stability in all domains of life.

# CHAPTER 6: Conclusion and future directions

In the first part of this project we wanted to understand the link between R-loop formation and hypernegative supercoiling and we constructed a conditional *topA rnhA gyrB*(Ts) mutant and found that depleting RNase HI activity inhibited supercoiling. We performed a suppressor screen and identified suppressor mutations that restored supercoiling and corrected the growth and segregation defects of *topA rnhA gyrB*(Ts) mutant. The following conclusions can be drawn from the first part of this project:

RNase HI plays an important role in regulating DNA supercoiling by modulating gyrase activity. Assays on gyrase activity in *vivo* and in crude cell extracts of *topA rnhA gyrB*(Ts) indicates that this modulation is via the ATP dependent supercoiling reaction of gyrase. In these assays the ATP dependent supercoiling reaction of gyrase was inhibited while the ATP independent relaxation was unaffected. This suggest that in the absence of RNase HI, an inhibitor is synthesized that specifically target only the ATP dependent supercoiling reaction of gyrase. In fact supercoiling analysis at 37ºC in a single *rnhA*$^-$ mutant revealed that DNA was more relaxed in this mutant compared to a wild type control and relaxation was more evident in *rnhA gyrB*(Ts) mutant compared to *rnhA*$^-$ alone (Usongo, unpublished results). This indicates that the effect of this inhibitor is more dramatic when gyrase is defective as is the case with *gyrB*(Ts) and when replication is unregulated as is the case in the triple *topA rnhA gyrB*(Ts) mutant.

RNase HI reduces unregulated replication to allow proper chromosome segregation prior to cell division. In *topA rnhA gyrB*(Ts) unregulated replication due to the absence of *topA* and *rnhA* both lead to the accumulation of stable R-loops. Replication from these R-loops (cSDR) in addition to replication from *oriC* actually overwhelms the capacity of the cell to segregate the chromosomes. A good number of suppressor mutations that restored supercoiling and corrected the growth and segregation defects of *topA rnhA gyrB*(Ts) cells were in genes that affected DNA replication and nucleotide metabolism. More importantly we also isolated suppressors that were shown to reduce cSDR (*rne*::*aph*, *rnr::aph* and *dnaT::aph*).

It is possible that the in the absence of RNase HI, the biochemical pathway that synthesizes ATP in the cell (Krebs cycle and glycolysis) might be affected limiting the amount of ATP available in the cell. This might explain why suppressors were mapped in genes affecting DNA replication and nucleotide metabolism. Since cellular processes such as replication consume a lot of ATP, reducing replication will make more ATP available for gyrase. In this case, it will be interesting to investigate the possibility that the absence of RNase HI renders the metabolic pathway for ATP synthesis defective. I propose that experiments be performed to shed more light on this possibility. To achieve this, the expression of genes implicated in the synthesis of ATP in the Krebs and glycolytic pathway will need to be analyzed by DNA microarray. The expression patterns in *rnhA*$^{-}$, *gyrB*(Ts), *topA rnhA gyrB*(Ts) and a wild type control strain will give us valuable information on this possibility.

In the first part of this project, our goals were to understand the mechanism of supercoiling inhibition in the triple *topA rnhA gyrB*(Ts) mutant and to subsequently identify the gyrase inhibitor. Our results from the suppressor screen provide interesting leads on the way forward to identify potential gyrase inhibitor(s). Supercoiling by gyrase was dramatically restored both *in vivo* and *in vitro* in three suppressor mutations (*ynjD*, *ygiL* and *ybgP*). The function of these genes in *E. coli* is still unknown and they are potential candidates for a gyrase inhibitor. Further experiments need to be performed to validate them as gyrase inhibitors. The suppressor mutations need to be transduced into a wild type strain and tested for supercoiling stimulation at various growth phases. The genes of validated candidates needs to be cloned and the protein overexpressed and purified. The purified protein will then be assayed on the ability to inhibit the supercoiling activity of gyrase *in vitro*.

The second part of this project was to study the roles of type IA topoisomerases in chromosome segregation and genome maintenance. The following conclusions can be drawn from the second part of this project.

Type IA topoisomerases play a role in chromosome segregation via supercoiling by controlling the number of forks travelling on the chromosome and this is achieved by limiting firing from *oriC*. In the absence of *topA*, excess supercoiling will promote DNA

melting at the origin leading to unregulated replication. Moreover, one major function of *topA,* a type IA topo is to prevent R-loop formation that leads to cSDR. In a *gyrB*(Ts) strain at the nonpermissive temperature, deleting *topA* corrects the replication initiation problem via supercoiling. Because gyrase is defective, the unregulated replication from *oriC* will make this defective gyrase unable to support chromosome segregation and this will render topo III indispensable for the execution of this function.

Type IA topoisomerases play a role in genome maintenance by controlling initiation from *oriC* and by destabilizing R-loops. R-loops can also trigger fork collisions outside of the *ter* region and this has been shown to be an important source of genomic instability in *E. coli* (Wimberly et al., 2013). The control of replication in bacteria is of great importance because of their short generation time whereby new rounds of replication are started before the completion of previous rounds, implying that all the stages of replication must be tightly regulated to ensure proper chromosome segregation and cell division. Type IA topoisomerases thus contribute to this regulation by controlling initiation from *oriC* and by destabilizing R-loops that act as alternative sources of replication independent of the tightly controlled and regulated *oriC*/DnaA system.

As is the case in eukaryotes, bacterial type IA topoisomerases also play a role in genome maintenance by acting with RecQ to prevent RecA-dependent chromosome segregation defects. Unlike in eukaryotes where it is well established that RecQ acts with Top3, in *E. coli* we provide genetic evidence that RecQ acts with topo I to prevent RecA-dependent chromosome segregation defects that will otherwise lead to genome instability. In fact it has been shown that *E. coli* topo I can compensate for the absence of Top3 in *S. cerevisiae* (Wallis et al., 1989; Gangloff et al., 1994). Topo I is present in all bacteria while topo III is present only in a limited set of bacteria. This seems to suggest that in bacteria whereby only topo I is present, it will substitute for the function of topo III. It is possible that topo I in these bacteria have evolved to be efficient in decatenation and precatenane removal (Li et al., 2000). I proposed that experiments be performed on bacterial species which have only topo I. The *in vitro oriC*-based replication system (Hiasa et al., 1994; Hiasa & Marians, 1994a) can be used to assess whether topo I from these bacteria can perform functions assigned to topo III in this system. It is reasonable to speculate that in

bacteria, topo III acts as a back-up for the more abundant topo I and this may explain why cells lacking topo III activity display no obvious phenotypes (Digate & Marians, 1989).

# CHAPTER 7: References and Annexes

# 7.1. References

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## 7.2 Annex I: Table showing suppressors of triple *topA rnhA gyrB*(Ts) mutant

| Number of independent isolates | Category | Comments |
|---|---|---|
| 28 | Reduce replication | *holC*(8): clamp loader, interaction with SSB, processivity<br>-*oriC, dnaT* (replisome loading, cSDR)<br>-nucleotides metabolism (18):<br>affect dNTP pool; some also increase ATP pool (supercoiling defect was corrected)<br>-The findings reported in this thesis demonstrate the correlation between the ability to reduce replication and supercoiling restoration. |
| 16 | Ribonucleases | *rne* (12). Corrects supercoiling by reducing cSDR. Our data demonstrates correlation between this category of mutants and the reduction of cSDR<br>*rnr* (4). Correction of supercoiling defect by RNase R possibly linked to cSDR. |
| 7 | Implicated in the formation of type I fimbriae | Fimbriae synthesis affects swarming/swimming make more ATP available for gyrase (very high supercoiling activity observed in this category of suppressors. Prominent in this category is the gene *fimD*. |
| 5 | Global regulators | *cyaA* and *crp* (5). Global transcription regulator that controls the expression of more than 200 genes. Exert global control over processes of cellular physiology and virulence such as catabolite repression and motility (type I fimbriae formation). Supercoiling was restored in these mutants and this could be related to ATP availability. |
| 2 | Central metabolism | *pta* and *aceE*<br>Supercoiling defect was corrected in these mutants. These genes are involved in central metabolism. Correlation between central metabolism and DNA replication has been established. Reduction of acetyl-CoA pool in *aceE* mutants affects flagella movement via CheY acetylation. In both instances more ATP might be made available for supercoiling and this might explain the correction of the supercoiling defect. |
| 8 | others | In this category we had genes of unknown functions. Supercoiling defect was corrected in mutants of this category. Some of them were putative ATPase's. Very high supercoiling activity was observed in three genes in this category: *ynjD*, *ygiL*, and *ygbP*. |

## 7.2. Annex II: Published article not discussed in this thesis

In the course of my PhD work, I also participated in the publication of an article that is not mentioned in this thesis. I was the second author in this publication. My contribution in this project was significant. I performed all the plasmid supercoiling experiments that established the correlation between the relaxation of hypernegative supercoiling and growth resumption. This article can be viewed using the link below.
http://www.ncbi.nlm.nih.gov/pubmed/18790862.