

Article

Role of Supercoiling and Topoisomerases in DNA Knotting

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Abstract: DNA knots are deleterious for living cells if not removed. Several theoretical and simulation approaches address the question of how topoisomerases select the intermolecular passages that preferentially lead to unknotting rather than to the knotting of randomly fluctuating DNA molecules, but the formation of knots in vivo remains poorly understood. DNA knots form in vivo in non-replicating and replicating molecules, and supercoiling as well as intertwining are thought to play a crucial role in both the formation and resolution of DNA knots by topoisomerase IV. To confirm this idea, we used two-dimensional agarose gel electrophoresis run with different concentrations of chloroquine to demonstrate that non-replicating pBR322 plasmids grown in a topoisomerase I-defective *E. coli* strain (RS2λ) were more negatively supercoiled than in a wild-type strain (W3110) and, concurrently, showed significantly fewer knots. In this way, using wild-type and *E. coli* mutant strains, we confirmed that one of the biological functions of DNA supercoiling is to reduce the formation of DNA knots.

Keywords: DNA topology; DNA knots; supercoiling; topoisomerases; two-dimensional agarose gel electrophoresis



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1. Introduction

The circular genome of the polyoma virus was the first in which supercoiling was observed [1]. Supercoiling is the coiling of a DNA molecule beyond its normal helical structure, either in a positive or negative direction, resulting from being over- or under-winded [2]. Topoisomers are the different forms that covalently closed circular (CCC) DNA molecules can adopt without changing its mass. The finding that DNA can be supercoiled, coinciding with the discovery of topoisomerases [3], led to a branch of biology called DNA topology, focusing on the study of DNA's structural properties [4].

Due to the helical structure of the DNA's backbone, once circularized, the double helix strands cannot be separated without breaking (at least one of them). They are topologically linked, with the linking number (Lk) representing the twists in the original DNA molecule if it was relaxed. Lk, after circularization, must be an integer. This property is fundamental in three-dimensional space, where covalently closed curves (CCCs) exist. Lk depends on two geometrical properties of DNA: twist (Tw) and writhe (Wr). Tw describes how the individual strands of DNA coil around the axis of the DNA helix and writhe describes how the helix axis coils in space. Because Lk is invariant for any given CCC, any change in the Tw of the molecule must be accompanied by an equal and opposite change in Wr, and vice versa [5]. A DNA molecule is said to be negatively (−) supercoiled when the Lk is lower than in the relaxed circular DNA of the corresponding size [6]. Both in vivo [7] and in vitro [8], (−) supercoiled bacterial plasmids are known to adopt a right-handed intertwined configuration in which the duplex–duplex crossings have a (−) sign (clockwise direction).

Topoisomerases are enzymes that can change DNA topology and, as such, allow non-continuous deformations of DNA molecules involving transient opening and sealing of covalent bonds in the DNA backbone. Therefore, DNA topoisomerases interconvert different topological states of DNA [9]. In *Escherichia coli*, both the *topA* gene and the *topB* gene encode type 1 topoisomerases (Topo I and Topo III, respectively). These enzymes make single-strand breaks in DNA and facilitate changes in Lk changes in increments of one [10]. The main function of Topo I is to survey the level of DNA torsional tension resulting from negative supercoiling by DNA gyrase. To fulfill this function, Topo I is activated when the DNA is strongly (–) supercoiled [11]. Topoisomerase III is dispensable for cell viability and has no known role in DNA supercoiling, but its absence triggers a mutator phenotype [12]. Bacterial type 2 topoisomerases, DNA gyrase, and Topoisomerase IV (Topo IV), are indispensable for cell viability [13]. These type-2 enzymes make double-stranded DNA breaks and alter the Lk of DNA in steps of two. The genes encoding the Topo IV subunits are *parC*, which is homologous to *gyrA* of gyrase, and *parE*, which is homologous to *gyrB* of gyrase. Gyrase introduces (–) supercoils, which are required for the initiation of replication [14] while Topo IV removes precatenanes behind the replication fork [15–17] and also knots [18].

As soon as DNA topoisomerases were discovered, it was shown that DNA knots could form in living cells. James Wang and co-workers observed for the first time knots in single-stranded DNA [19]. The simplest type of knot that can be made in closed-circular DNA, or any other closed curve, is a trefoil knot (or three-node knot).

DNA replication, transcription, and recombination are facilitated by type-2 DNA topoisomerases that catalyze passages of double-stranded DNA segments through each other. While the possibility of passing DNA segments through each other is generally beneficial, it may lead to the creation of DNA knots that have potentially devastating effects for cells if not removed efficiently [20]. DNA knots form in vivo in non-replicating cells [21,22] and also during replication [23–26]. Although type-1 DNA topoisomerases and DNA gyrase can knot and unknot DNA duplexes in vitro [27], there is enough evidence that Topo IV is the one involved in decatenation and unknotting bacterial DNA in vivo [18,28], and it was proposed that it is also the topoisomerase that makes knots during DNA replication [29]. López and co-workers proposed that when replication forks slow down or stall, sister duplexes become loosely intertwined and, under these conditions, Topo IV could inadvertently make the strand passages that lead to the formation of knots. This approach could also explain the observation that DNA gyrase inhibitors increase the content of knots in non-replicating bacterial plasmids [21,22]. On the other hand, Burnier's numerical simulations proposed that DNA supercoiling inhibits DNA knotting [30].

Here, we wanted to analyze how DNA knotting is influenced by DNA supercoiling in non-replicating pBR322 to test if DNA knotting by Topo IV depends on the level of DNA supercoiling. Firstly, we confirmed that pBR322 isolated from an *E. coli* strain (RS2λ) carrying a mutation in the *topA* gene, which reduces Topo I activity 100-fold, showed more (–) supercoiled DNA than in the wild-type strain [7]. Afterwards, we validated the simulation of Burnier and co-workers. Plasmids grown in the RS2λ strain showed less knotted molecules than in the wild-type strain. We performed these experiments by two-dimensional (2D) agarose gel electrophoresis. Agarose gel electrophoresis is the most commonly used method for the separation of linear DNA molecules as a function of their size. One-dimensional agarose gel electrophoresis, however, is not always able to separate DNA molecules of the same mass but differing in their conformation (i.e., knotted molecules). To resolve this problem, 2D agarose gel electrophoresis was developed in 1987 to analyze plasmid DNA replication and topology. This technique consists of two consecutive electrophoreses where the second dimension occurs perpendicular to the first. In addition, the conditions employed during the first dimension minimize the effect of molecular shape on electrophoretic mobility, whereas this effect is maximized during the second dimension [31]. The electrophoretic behavior of knotted molecules was further analyzed using 2D agarose gel electrophoresis [32,33].

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, and Culture Medium

The *E. coli* strains used in this study are W3110 [18], and RS2λ [7]. Competent cells were transformed with monomeric forms of plasmid pBR322 [34]. Cells were grown in LB medium at 37 °C. In both cases, 75 µg/mL ampicillin was added to the LB medium. Isolation of plasmid DNA was performed as described recently [35,36].

2.2. DNA Treatments

DNA was digested with Nb.BsmI or Nb.BtsI (New England Biolabs, Ipswich, MA, USA) for 1 h at 50 or 37 °C, respectively, to induce single-stranded breaks and, then reactions were blocked with 100 µg/mL proteinase K (Roche, Basel, Switzerland) for 30 min at 37 °C.

2.3. 2D Agarose Gel Electrophoresis and Southern Transfer

The first dimension occurred in a 0.4% Seakem[®] LE agarose (Lonza Rockland, Inc., Rockland, ME, USA) gel in TBE buffer (89 mM Tris-borate, 2 mM EDTA) at 0.9 V/cm at room temperature for 22 h. The second dimension took place in a 1% agarose gel in TBE buffer and was run perpendicular to the first dimension. The dissolved agarose was poured around the excised agarose lane from the first dimension. The second dimension was at 5 V/cm in a 4 °C cold chamber for 9.30 h. When it was necessary to resolve topoisomers, different concentrations of chloroquine (CHL) (Sigma, St. Louis, MO, USA) were added to the TBE buffer in both the agarose gel and the running buffer. The time to run this type of 2D agarose gel electrophoresis was slightly longer: 25 h in the first dimension and 10 h in the second dimension. Southern transfer was performed as described previously [35,36].

2.4. Non-Radioactive Hybridization

Non-radioactive hybridization was performed as described previously [35,36].

2.5. Analysis of the Topology of CCCs

DNA Lk was calculated by quantifying the amount of every given topoisomer of CHL 2D gel immunograms using the Image J64 software. DNA supercoiling density (σ) was calculated according to the equation $\sigma = \Delta Lk / Lk_0$. The linking number difference was calculated using the definition $\Delta Lk = Lk - Lk_0$. In this case, $Lk_0 = 4363 \text{ bp} / 10.5 \text{ bp/turn} = 415$ for pBR322 [37].

To calculate the ΔLk generated by any specific CHL concentration, we performed 2D agarose gel electrophoresis, adding CHL only during the second dimension, and counted the number of signals between the topoisomer that had a $\Delta Lk = 0$ before the first dimension to the topoisomer that migrated with a $\Delta Lk = 0$ after the second dimension.

3. Results

3.1. Experimental Approach

In pBR322, transcription of the constitutive tetracycline-resistance gene occurs against the direction of the replication fork movement (Figure 1). This affects supercoiling and leads to the formation of a significant number of knotted molecules [21].

As pointed out in the Introduction, supercoiling relies on the activity, processivity, and proper function of DNA gyrase, which introduces (–) supercoiling, and Topo I activity, which removes (–) supercoiling. Firstly, we transformed a wild-type *E. coli* strain (named the W3110 strain) with pBR322 to be used as a control. On the other hand, *topA10 E. coli* cells (RS2λ strain) carrying a mutation in the *topA* gene, which reduces Topo I activity 100-fold [38], were transformed with pBR322 to study its level of supercoiling.

Then, plasmid DNA was isolated from exponentially growing W3110 and RS2λ cells and analyzed by 2D agarose gel electrophoresis.

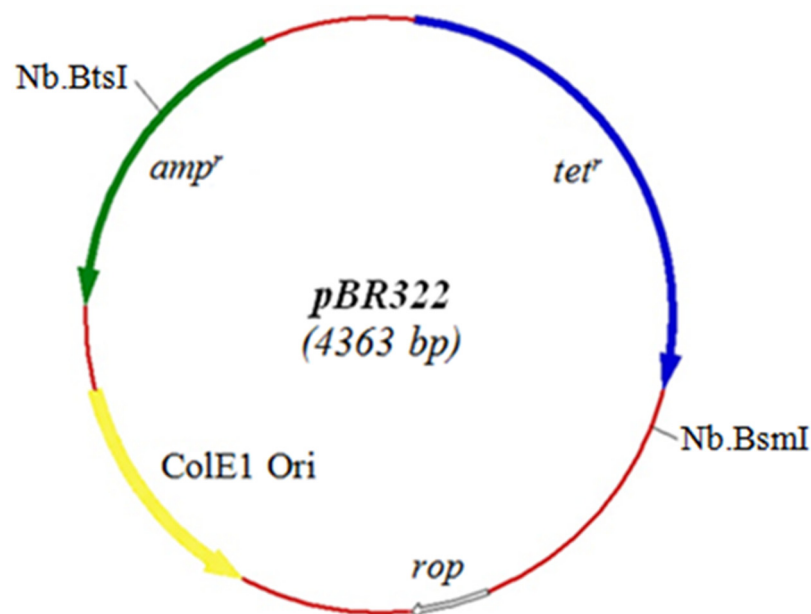


Figure 1. Map of pBR322. Map showing the relative position and orientation of its most relevant features: the unidirectional replication origin ColE1 (yellow arrow), the *rop* gene (white arrow) that encodes the homodimeric protein involved in regulation of the plasmid copy number, and the ampicillin- and tetracycline-resistance genes (green and blue arrows, respectively). The cleavage sites of enzymes Nb.BtsI and Nb.BsmI are marked on the map.

3.2. Identification of Supercoiling Density (σ) by Chloroquine 2D Agarose Gel Electrophoresis

To determine the supercoiling density, we need to identify all the topoisomers of each population. To resolve the abundance of each topoisomer, we used a procedure adapted from the Brewer–Fangman 2D agarose gel electrophoresis system [31,39] based on adding chloroquine (CHL) to both the agarose gel and the electrophoretic buffer. CHL is a planar molecule that can intercalate between the two strands of the DNA double helix. This intercalation causes a reduction in DNA twists. As in vivo bacterial plasmids are negatively supercoiled, increasing concentrations of the drug progressively remove the negative supercoiling first, and add positive supercoiling only after all the native negative supercoiling has been removed. Combining 2D agarose gel electrophoresis with CHL (CHL 2D gel) results in a very powerful resource to resolve topoisomers of covalently closed circular (CCC) plasmid populations [40,41]. Taking into account the CHL concentration added to the 2D agarose gel electrophoresis, it is possible to calculate the most abundant topoisomer (the mode).

After several attempts, we decided to use different concentrations of CHL during both the first and the second dimensions. In the case of plasmids isolated from W3110 cells, the first dimension was performed with 1 $\mu\text{g/mL}$ CHL and the second dimension in the presence of 2 $\mu\text{g/mL}$, whereas for plasmids isolated from RS2 λ cells, it was necessary to use 2 $\mu\text{g/mL}$ CHL during the first dimension and 5 $\mu\text{g/mL}$ during the second. As plasmids isolated from RS2 λ cells initially had a lower σ than plasmids isolated from W3110 cells, higher concentrations of CHL were needed.

Figure 2 shows the immunograms corresponding to 2D agarose gel electrophoresis of pBR322 isolated from W3110 (Figure 2A) and RS2 λ cells (Figure 2B). The immunograms were aligned so that nicked (open circle, OCs) and linear forms (Ls), which do not significantly change their electrophoretic mobility in the presence of different concentrations of CHL, coincided. In this way, it was easier to compare the electrophoretic mobility of different topoisomers [25]. To quantify the abundance of every topoisomer by densitometry we used different exposures of the films and analyzed them with Image J64 software. In the case of plasmids isolated from W3110 cells, the topoisomers representing the mode rose

to $\Delta Lk = -26$ and $\sigma = -0.062$, whereas in the case of plasmids isolated from RS2 λ cells, these values were $\Delta Lk = -31$ and $\sigma = -0.074$.

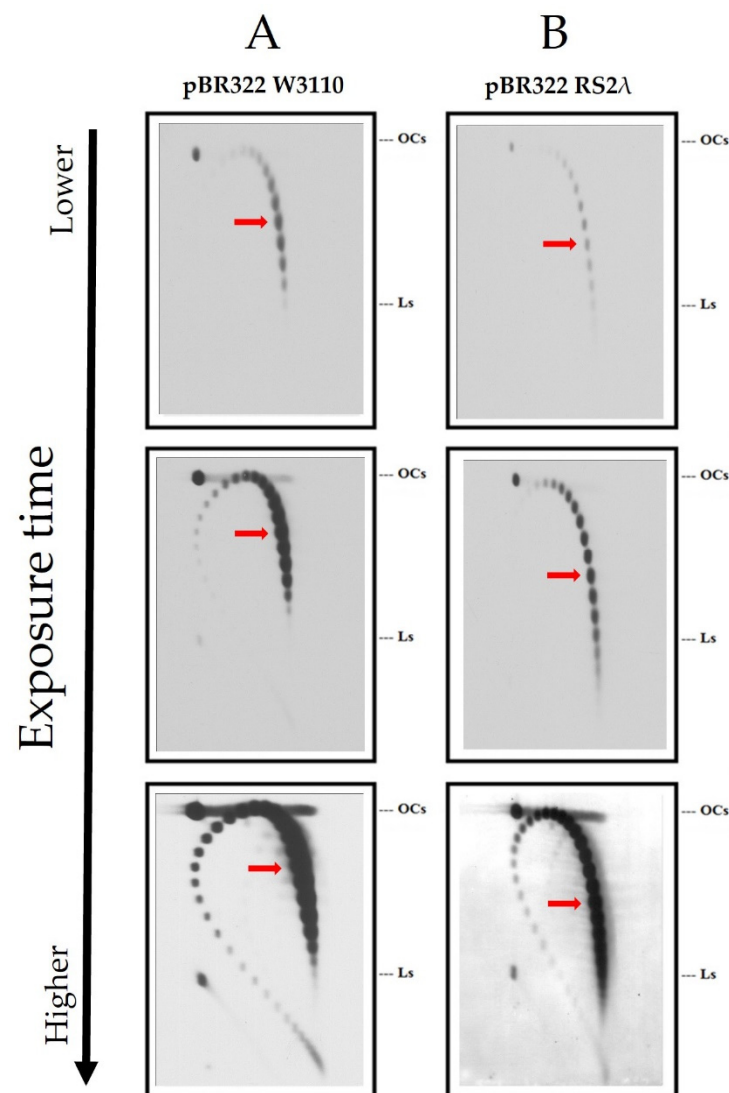


Figure 2. Immunograms (with different exposures times) corresponding to pBR322 populations' DNA isolated from W3110 and RS2 λ strains, analyzed by 2D agarose gel electrophoresis performed with CHL to observe the different topoisomers. Red arrows indicate the most abundant topoisomer (mode) in each population. OCs = open circle forms and Ls = linear forms. In all cases, the first dimension was carried out for 25 h and the second dimension was carried out for 10 h. **(A)** Immunograms corresponding to the population grown in the W3110 strain. The first dimension was carried out with 1 $\mu\text{g/mL}$ of chloroquine and 2 $\mu\text{g/mL}$ was used in the second dimension. **(B)** Immunograms corresponding to the population grown in the RS2 λ strain. The first dimension was carried out with 2 $\mu\text{g/mL}$ of chloroquine and the second dimension with 5 $\mu\text{g/mL}$.

3.3. Analysis of Knotted Molecules by 2D Agarose Gel Electrophoresis

Once the level of supercoiling was evaluated in plasmids isolated from both strains, the next step was to study the proportion of knots formed in both populations. In untreated samples, the knots were masked by supercoiling, making it impossible to analyze them. In order to analyze DNA knots in a gel, DNA supercoiling must be removed [33,42]. A key feature of closed circular molecules is their topological linkage, requiring strand separation by breaking one of their strands [43]. For this reason, plasmid DNA was nicked with a nicking endonuclease to remove all supercoiling, and CCC molecules were converted to

OC (Figure 3), unless the topoisomer had a knot. In such cases, the topoisomers migrate into the gel according to the number of crossings of the knot [27,32].

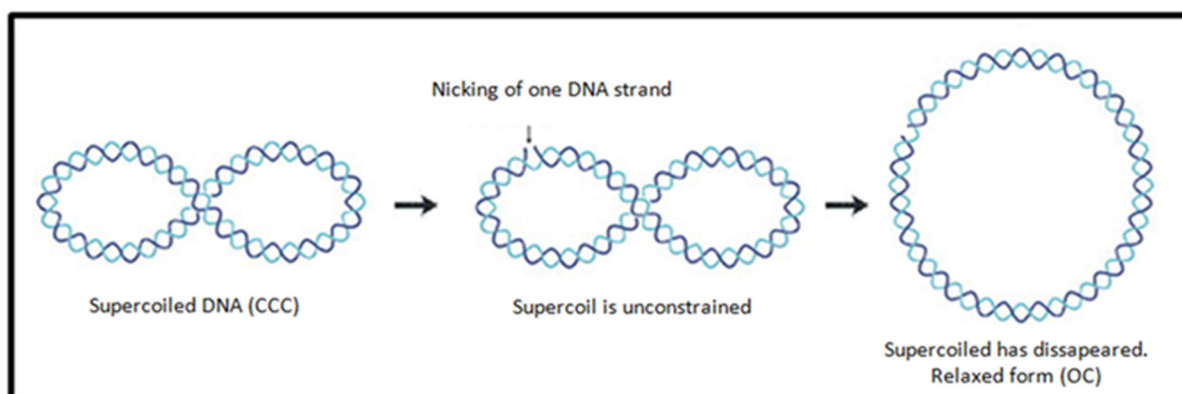


Figure 3. Representative diagram of the CCC and OC forms which can take a circular DNA molecule. When a CCC molecule is nicked, changing the linking number causes one of the two polynucleotide chains to revolve on the other by releasing the tension at the ends, turning CCC molecules into OC molecules. CCC = covalent closed circle and OC = open circle.

We can identify different molecules of our plasmid DNA using 2D agarose gel electrophoresis (Figure 4A,B). In pBR322 isolated from W3110 cells without treatment with a nicking endonuclease (Figure 4A), the signal appearing at the bottom right of the immunogram corresponds to the supercoiled forms of the non-replicated monomers, the CCCs. Some of these molecules can suffer a single-stranded break during DNA isolation, during the first and second dimensions, and/or between the first and the second dimensions (displaying a horizontal and vertical trailing, respectively). Both trailings create a right angle (90°) between CCCs and OCs. At the apex of the right angle, a very faint discrete signal is detected, corresponding to molecules that suffered nicking between the first and second dimensions. Between the signals corresponding to OCs and CCCs, there are three arcs. These discontinuous arcs of signals are interpreted as topoisomers of the monomeric forms. The discontinuous nature of the arcs indicates that the individual molecular species differed in their ΔLk . Another signal that appears below the OC signal, at the bottom of the immunograms, corresponded to linear forms. Double-stranded breaks can also occur during DNA isolation, although less frequently, and they convert circular plasmids into linear molecules of the same mass regardless of the site where the break occurred. Figure 4B, corresponding to the same DNA sample treated with the nicking endonuclease (Nb.BsmI) before running the gel, shows the disappearance of the signal corresponding to the CCCs, indicating that nicking was complete, so that these molecules lost all their W_r and were converted to OCs or to knotted molecules. Knots with an odd number of crossings appeared with greater intensity than those knots with an even number of crossings. To study the proportion of knots in these plasmids, we compared, using densitometry, knotted molecules against OC signals (Figure 4C). The average of the densitometric profiles obtained from three different experiments was 26%.

Figure 5A,B show the immunograms of pBR322 molecules isolated from RS2 λ cells and analyzed by 2D agarose gel electrophoresis. In Figure 5A, it is possible to observe the same signals as in the previous case with one difference: the absence of two arcs of topoisomers between the CCCs and OCs. When the sample was nicked with a nicking endonuclease (Nb.BtsI), we could identify the arc corresponding to knotted molecules alone (Figure 5B). As in the case of the W3110 strain, knots with an odd number of crossings appeared with greater intensity than the knots with an even number of crossings. With Image J64, we determined the densitometry corresponding to the OC signals and knotted plasmids from all three different experiments (Figure 5C). The proportion obtained in this case was 9%.

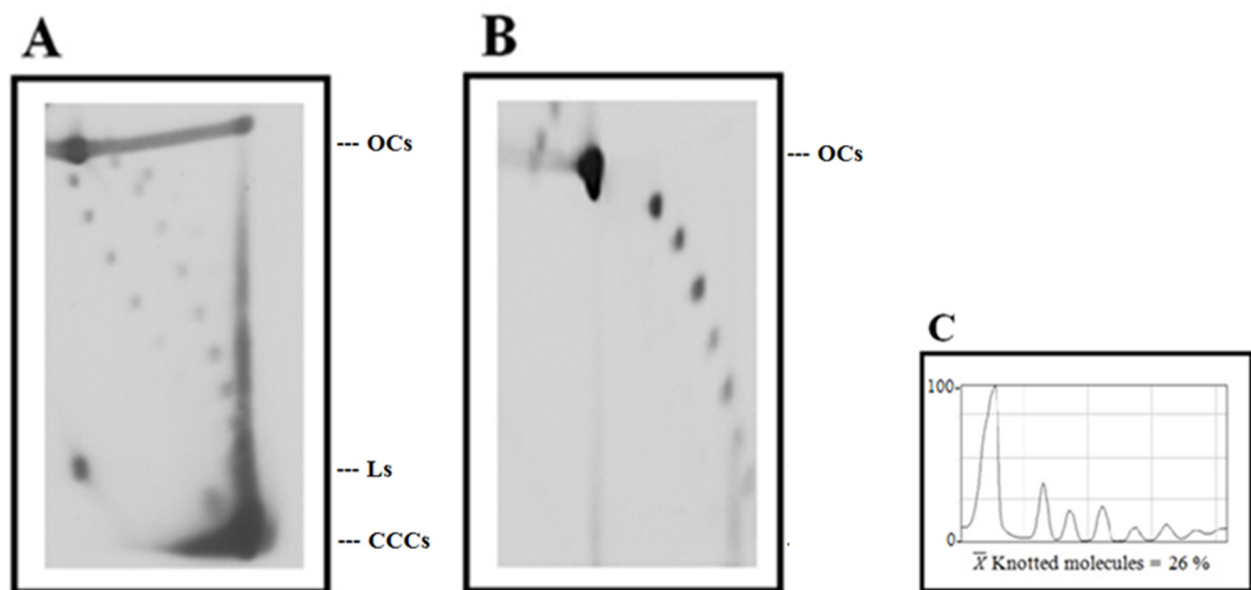


Figure 4. Analysis of knotted forms of pBR322 DNA isolated from the W3110 strain by 2D agarose gel electrophoresis. (A) Immunograms corresponding to intact forms of pBR322 isolated from *E. coli* W3110 cells. (B) Immunograms corresponding to nicked forms of pBR322 isolated from W3110 cells treated with Nb.BsmI. OCs = open circle forms, Ls = linear forms, and CCCs = covalent close circles. (C) Densitometric profile corresponding to the OC signal and knotted molecules of immunogram (B) with the percentage of knotted molecules calculated from all three different experiments.

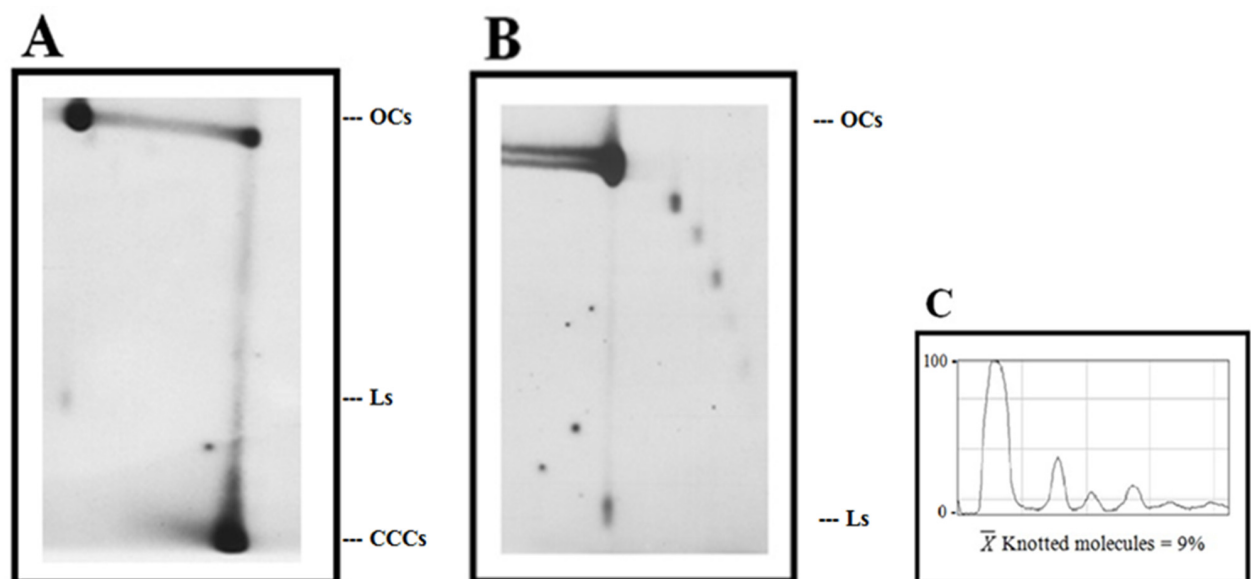


Figure 5. Analysis of knotted form of pBR322 DNA isolated from the RS2λ strain by 2D agarose gel electrophoresis. (A) Immunograms corresponding to intact forms of pBR322 isolated from *E. coli* RS2λ cells. (B) Immunograms corresponding to nicked forms of pBR322 isolated from RS2λ cells treated with Nb.BtsI. OCs = open circle forms, Ls = linear forms, and CCCs = covalent close circles. (C) Densitometric profile corresponding to the OC signal and knotted molecules of immunogram (B) with the percentage of knotted molecules calculated from all three experiments.

4. Discussion

What are the roles of topoisomerase in DNA knotting (and unknotting)? Is the level of supercoiling critical for the formation of knots by Topo IV? Does DNA supercoiling help Topo IV to keep the DNA unknotted in living bacterial cells?

When circular DNA molecules are isolated from bacteria, they are rarely knotted unless the host strain is defective in DNA gyrase [21] or Topo IV [44], or when gyrase inhibitors are added, as shown by Ishii and co-workers [22]. In Ishii's work, the use of gyrase inhibitors, such as oxolinic acid, increases the number of knotted molecules in pBR322. The authors highlight DNA gyrase as possibly being responsible for DNA knotting, as Topo IV was discovered only one year earlier [45]. The fact that inhibiting DNA gyrase showed a higher number of knots may suggest that DNA supercoiling may favor unknotting. Numerical simulations have linked DNA supercoiling to DNA knotting with different outcomes; Podtelezhnikov et al. suggests that negative supercoiling promotes DNA knotting [46], whereas Burnier et al. propose the opposite [30]. Additionally, modeling studies by Stasiak's lab point to a primary role of supercoiling in the removal of DNA knots [47–49].

Topo IV has been proposed to knot and unknot sister duplexes during DNA replication [29]. According to this work, when progression of the replication forks is impaired, sister duplexes become loosely intertwined. Under these conditions, Topo IV inadvertently makes the strand passages that lead to the formation of replication knots and removes them later to allow their correct segregation.

Topoisomerase II (Topo II) has been shown to play a role in DNA knotting by introducing small amounts of DNA knots within the clusters of nucleosomes of eukaryotic chromatin [50].

How are transcription and DNA knotting linked? Portugal and Rodriguez-Campos showed that T7 RNA polymerase can transcribe unknotted plasmids but cannot transcribe highly knotted plasmids [51]. In another recent study, Valdés et al. examined how transcriptional supercoiling of non-replicating DNA affects the occurrence of knots. They found that the accumulation of positive supercoiling generated in front of the transcribing complexes increases DNA knot formation [52].

In this study, we examined the level of DNA supercoiling and DNA knotting on non-replicating molecules using 2D agarose gel electrophoresis. Our results confirm that (i) increasing the concentration of chloroquine leads to a gradual decrease in the σ of the molecules [53]; (ii) plasmids isolated from RS2 λ cells have more (–) supercoiled DNA than plasmid isolated from wild-type cells [7]; and (iii) plasmids isolated from RS2 λ cells present less DNA knots than those isolated from wild-type cells, suggesting that negative supercoiling inhibits DNA knotting (this study).

5. Limitations

Our experiments were performed in pBR322 plasmids. It is not always feasible to extrapolate the observations made on small plasmids to bacterial or eukaryotic chromosomes. Plasmids are small topological domains that do not necessarily reflect the conditions of the large domains of the chromosomes of prokaryotes and eukaryotes.

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