Bacteriophage 4, e27517; January–December 2014; © 2014 Landes Bioscience

Effect of supercoiling on the λ switch

Kamilla Norregaard,[1] Magnus Andersson,[1,†] Kim Sneppen,[1] Peter Eigil Nielsen,[2] Stanley Brown,[1] and Lene B Oddershede[1,*]

[1]The Niels Bohr Institute; University of Copenhagen; Copenhagen, Denmark; [2]Department of Cellular and Molecular Medicine; Faculty of Health and Sciences; Copenhagen, Denmark

[†]Current affiliation: Department of Physics; Umeå University; Umeå, Sweden

The lysogenic state of the λ switch is exceptionally stable, still, it is capable of responding to DNA-damage and rapidly enter the lytic state. We invented an assay where PNA mediated tethering of a plasmid allowed for single molecule investigations of the effect of supercoiling on the efficiency of the epigenetic λ switch. Compared with non-supercoiled DNA, the presence of supercoils enhances the CI-mediated DNA looping probability and renders the transition between the looped and unlooped states steeper, thus increasing the Hill coefficient. Interestingly, the transition occurs exactly at the CI concentration corresponding to the minimum number of CI molecules capable of maintaining the pRM-repressed state. Based on these results we propose that supercoiling maintains the pRM-repressible state as CI concentration decline during induction and thus prevent autoregulation of *cI* from interfering with induction.

Keywords: supercoiling, λ switch, epigenetics, PNA, cooperativity, CI protein, tethered particle motion

Submitted: 11/08/2013

Revised: 12/12/2013

Accepted: 12/12/2013

http://dx.doi.org/10.4161/bact.27517

*Correspondence to: Lene B Oddershede; Email: oddershede@nbi.dk

Addendum to: Norregaard K, Andersson M, Sneppen K, Nielsen PE, Brown S, Oddershede LB. DNA supercoiling enhances cooperativity and efficiency of an epigenetic switch. Proc Natl Acad Sci U S A 2013; 110:17386–91; PMID:24101469; http://dx.doi.org/10.1073/pnas.1215907110

Introduction

The lysis–lysogeny decision by bacteriophage λ was the first genetic switch to be deciphered,[1] this epigenetic switch is now relatively well understood and the most important features are outlined in **Figure 1A and B**. One fascinating feature of this switch is the profound stability of the prophage state. The λ prophage responds to the host (*Escherichia coli*) DNA-damage sensing or SOS system. When the SOS pathway is induced, activated RecA protein catalyzes the self-cleavage of λ repressor protein, CI, causing the prophage to enter lytic development. In strains lacking RecA protein, three quarters of the released phage bear mutations in the *cI* gene.[2] Thus, the prophage state maintained by CI is more stable than the genes encoding components maintaining the repressed state. In fact, two more recent studies found 73 out of a total of 74 apparently wild type phage (cI^+) released from *recA* lysogens, were mutants in the promoter for repressor maintenance, pRM.[3,4] The rarity of wild type phage released from *recA* lysogens, as pointed out by Little and Michaelowski,[4] may not even be true escapees but may be the result of a mutation in the now dead host. The stability of the prophage state is in part due to the sophisticated regulation of CI synthesis (as detailed in **Fig. 1B**). The intracellular concentration of CI must be carefully fine-tuned such that it is high enough to maintain repression of lytic development but not too high to prevent sufficient degradation by activated RecA.[5] In the lysogenic state, CI activates its own synthesis from pRM.[6] At physiological concentrations CI can repress its own synthesis by binding to the operator, OR3, only if another segment of the prophage genome containing the operator OL3 is clamped next to OR3. The clamp is formed by CI binding as an octamer to the operators OL1-OL2 and OR1-OR2, thus arranging the intervening DNA in a loop (as depicted in **Fig. 1B**).[7] In other words, the OL-CI-OR looped state is necessary for CI repression of pRM.

CI-mediated looping of DNA has been extensively studied in vivo and

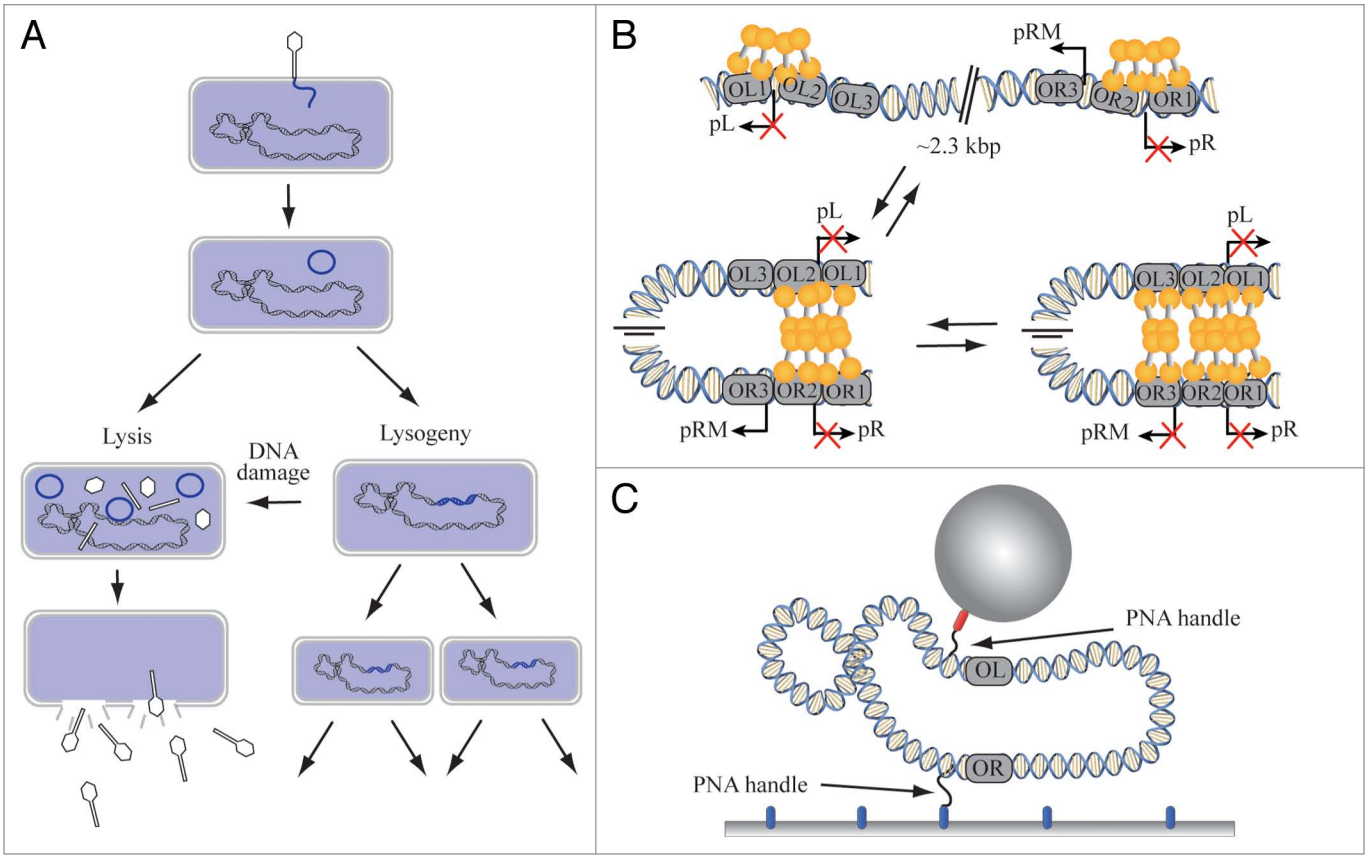

Figure 1. (**A**) λ phage survival strategies after infection of *Escherichia coli*. The phage enters either the lytic pathway (left) or the lysogenic pathway (right). The lytic pathway is irreversible and rapidly produces a crop of phage that is released by lysing the cell. The lysogenic pathway is a dormant state in which the phage DNA is incorporated into the host DNA and passively gets replicated until a signal (e.g., DNA damage) flips the switch and causes entering into the lytic state. (**B**) λ switch regulation. The λ operators, operator right (OR) and operator left (OL), are located ~2.3 kbp apart on the phage DNA overlapping the lysogenic (pRM) and lytic (pR and pL) promoters (marked by bent arrows). Each operator is a constellation of three adjacent sub sites that bind CI (yellow dumbbell) in a hierarchical manner. Cooperative binding between CI dimers bound at OR1 and the adjacent intrinsically weak operator OR2 virtually ensures simultaneously occupancy and is responsible for lytic repression (indicated by red cross) and simultaneously activation of the weak CI promoter pRM located at OR3. Long-range cooperativity affords increased stability to the lysogenic state by a CI octamer clamping OR1-OR2 and OL1-OL2 arranging the intervening DNA in a loop. This complex brings OL3 and OR3 in juxtaposition allowing a CI dimer bound on the intrinsic strong OL3 to assist a CI dimer binding at the weak OR3 resulting in pRM repression (marked by red cross). (**C**) The PNA-based TPM assay. A DNA plasmid is tethered between an anti-digoxigenin coated glass surface and a streptavidin coated polystyrene bead via digoxigenin/biotin PNA handles that form triplex invasion complexes with specific sequences on the DNA. The non-complementary strand is displaced as a small loop. The PNA handles are flanking the λ operators, OR and OL, which limit the λ immunity region.

in vitro. In vitro it was studied both in bulk and single molecule experiments,[8,9] where the most common single molecule approach employed was an assay where a linear DNA was tethered between a coverslip and a submicron sized bead whose Brownian motion was observed and analyzed. The larger the excursions of the particle, the longer the tether and the looped state (yielding an overall shorter DNA tether) could thereby be distinguished from the unlooped state. This type of single molecule assay relying on tethered particle motion (TPM) has confirmed the sites in OL and OR necessary for clamping.[9] Until recently, all TPM studies were performed with linear DNA with no supercoils unless they were mechanically introduced, e.g., by twisting a magnetic bead. Mechanically induced supercoils can be difficult to distinguish from protein-mediated looping events. In this addendum, we describe a novel TPM assay utilizing peptide nucleic acid (PNA) handles to tether natively supercoiled plasmid DNA.[10] We discuss, to our knowledge, the first single molecule evidence of the stability of the pRM repressible state at physiological CI levels and speculate what features of supercoiling may be responsible.

PNA Tethering of Supercoiled DNA

Recently, we developed a novel chemical/biological assay for investigating protein-mediated DNA looping at a single molecule level using naturally supercoiled DNA.[10] Our assay (shown in **Fig. 1C**) consists of a single supercoiled plasmid tethered by sequence specific PNA handles allowing us to tag points on circular DNA without introducing any breaks in the DNA. We attach the PNA handles such that they flank the λ immunity region, tethering the DNA molecule between a glass surface and a

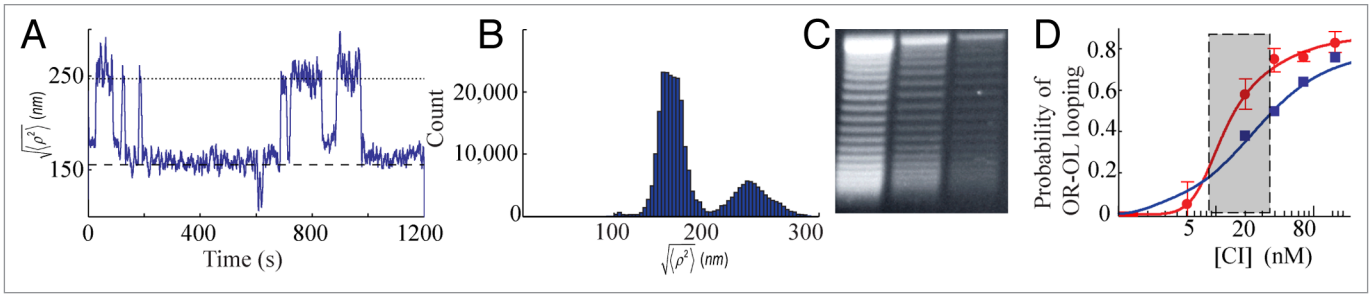

Figure 2. (**A**) The size of the thermal fluctuations of the plasmid tethered bead as function of time in presence of 80 nM CI. The unlooped state causes the bead to exhibit larger fluctuations (indicated by the dotted line) than the looped state (dashed line). (**B**) Histogram corresponding to the time series shown in (**A**), the left peak corresponds to the looped state, the right peak to the unlooped state. (**C**) Chloroquine gel containing 4-fold dilutions of the supercoiled plasmid preparation. The large number of bands confirms that there is a large spread in supercoils (writhe number) in the sample. (**D**) Probability of CI-mediated looping as function of CI concentration for supercoiled DNA (red circles) published in reference 10 and for linear DNA (blue squared) published by Zurla et al.[9] The lines show the corresponding thermodynamical models. Supercoiling enhances the binary response to changes in CI concentration and lowers the CI concentration necessary for pRM repression. The dashed box highlights the narrow sigmoidal transition interval for the supercoiled DNA. Interestingly, 20 nM CI (at the center of the transition interval) corresponds to 12 CI monomers available in the cell and this is exactly the number of CI molecules necessary to form a pRM repressible state. The number of data sets for each concentration is n = 4 for 5 nM CI, n = 7 for 20 nM CI, n = 5 for 40 nM CI, n = 6 for 80 nM CI, and n = 9 for 170 nM CI. Error bars represent one standard deviation.

submicron sized polystyrene bead. TPM experiments confirmed that we could distinguish the dynamics of supercoiled plasmids compared with that of relaxed plasmids and linear relaxed tethers.[10] Using this assay employing naturally supercoiled DNA, CI-mediated looping could be detected directly as a decrease in the Brownian motion of the tethered bead, thus producing a telegraph-like signal over time as shown in **Figure 2A**. We envision this PNA-based assay having numerous applications beyond studies of the λ switch. It could enable studies of protein/enzyme DNA interactions on supercoiled DNA mimicking the native bacterial DNA or eukaryotic chromatin, without the requirement of external interference, e.g., magnetic tweezers.[11-13] We caution interested researchers that in our TPM studies of supercoiled DNA we took great care with the purity of all reagents used: The plasmid DNA was purified through two rounds of isopycnic centrifugation with CsCl and ethidium bromide, the PNA was purified by HPLC and all solutions were filtered through several centimeter beds of fine sephadex to remove particles. We did not examine reagents purified by other methods and therefore cannot comment on their suitability for the supercoiled single molecule assay. We also manipulated all solutions containing PNA/DNA with low-binding and DNAase free plasticware.

Effect of Supercoiling on Looping Probability

Using the PNA-based single molecule assay, the probability of a DNA plasmid being in the looped state is determined by examining the thermal excursions of the tethered bead,

$$\sqrt{\langle \rho^2(t) \rangle_{20ms}},$$

as a function of time (see **Fig. 2A**). The corresponding histogram is shown in **Figure 2B** where the peak centered around 180 nm corresponds to the looped state and the peak around 250 nm to the unlooped state.

Plasmid DNA as recovered from bacteria contains a varying number of supercoils, the writhe number of any single plasmid molecule being constant throughout the looping experiments. To examine the distribution in writhe number, we electrophoresed a sample through a chloroquine gel as shown in **Figure 2C**. The large variety in writhe between plasmids with identical sequence and length posed a challenge to the interpretation of the looping dynamics because the magnitude of the Brownian motion from, say, an unlooped state would vary from one tethered bead to another. Also, this meant that a calibration curve relating sequence length to magnitude of Brownian fluctuation (as routinely done in the linear assays[14]) did not make sense for the supercoiled assay. To overcome this challenge, for each individual time series (as shown in **Fig. 2A**) we calculated the histogram (**Fig. 2B**) and assigned the lower peak to the looped state and the higher peak to the unlooped state. This assignment helped normalize all data sets and collapse them into a single histogram. The ratio of the area under the peak representing the looped state to the total area was then assigned as the probability of looping. This procedure was performed at different CI concentrations, thus establishing the looping probability as function of CI concentration for a supercoiled assay as shown in **Figure 2D** (red circles).

Finzi and coworkers examined CI-mediated looping probabilities using a linear DNA assay in the physiologically relevant CI concentration regime.[9] Their results are shown by blue squares in **Figure 2D**. In the linear assay, the DNA looping probability curve can be described by a low Hill coefficient (h = 1.2). This means the repressible state of pRM is continually responsive to changes in CI concentration and as CI is depleted with activated RecA, pRM will compensate by trying to produce more CI. Considering the sophistication of the prophage maintaining system, such counteraction of CI regulation against lytic induction would be surprising. We speculate that the observed low Hill coefficient was due to the relaxed

nature of linear DNA. This is supported by the results utilizing the supercoiled assay, where we found that the response of supercoiled DNA to varying concentrations of CI was very different from the response of linear or relaxed DNA[10] (compare red circles to blue squares in **Fig. 2D**). Instead of observing a continuous response to CI concentration, the supercoiled assay exhibited a sharp transition between the unlooped and looped state, the narrow transition interval is highlighted by a dashed box in **Figure 2D**. At CI concentrations above approximately 25% of the average lysogenic concentration (~200 nM CI[15]) the operators are nearly always in the looped, pRM-repressible state. Only when the CI concentration falls below approximately 5% of the average lysogenic concentration do the operators spend the majority of their time in the unlooped, pRM-non-repressible state.

The Hill coefficient describing the looping probability of naturally supercoiled DNA as function of CI concentration was estimated to be h = 2.5 (in contrast to h = 1.2 from the linear DNA). Regulatory proteins rarely act alone but instead cooperatively as a multi protein complex. A higher Hill coefficient can be interpreted as a higher degree of cooperativity in a system. Cooperativity helps ensure efficient discrimination between two states and simultaneously enhances a switch-like response to small changes in regulator concentration. Recently it was shown in vivo that DNA looping could be abolished if CI cooperativity was eliminated, supporting the necessity of cooperativity for λ switch efficiency.[16] Therefore, the observed increased Hill coefficient of the supercoiled DNA is in agreement with the binary developmental nature of the λ switch.

Features of Supercoiling Possibly Responsible for Higher Hill Coefficient

DNA supercoiling is an intrinsic property of the circular *Escherichia coli* DNA that helps compact the DNA in a highly condensed form. Because of the topology of supercoiled DNA it can in itself be considered as a regulatory parameter for many cellular processes such as transcription and protein interactions. DNA supercoiling has been found to facilitate protein-protein and enhancer-promoter communication over large distances by increasing the concentration of DNA segments in vicinity to each other.[12,17,18] In addition, changes in the degree of supercoiling induced by a previous RNA polymerase can either help a subsequent RNA polymerase to enter directly into the open complex or impede the movement of a RNA polymerase.[19] Supercoiling is also known to facilitate unwinding of short DNA segments and thereby expose single stranded DNA to which regulatory proteins preferentially bind.[13] Supercoiling is also thought to affect gene regulation by changing the local structure and dynamics of DNA[20] which can influence protein-DNA binding by making the helical structure more or less accessible for the proteins. The possibilities are many and future investigations will be necessary to determine what aspect of supercoiled DNA is responsible for the higher Hill coefficient.

Thermodynamic Parameters

By analyzing our data in the light of a thermodynamic model put forward in refs.[7,21,22] we estimated the cooperative binding energies associated with loop formation. The model including the free energies for the supercoiled system is shown as the red curve in **Figure 2D**. In the model, ΔG_{oct} represents the net free energy change due to octamerization of CI bound across OL-OR together with the cost of formation of a DNA loop, this was set to 0 kcal/mol. ΔG_{tetr} which represents the cooperative free energy due to tetramerization of CI bound across OL3-OR3 was set to -1.0 kcal/mol. By setting ΔG_{oct} to 0 kcal/mol we require that supercoiling together with octamerization of CI balances the cost of bending the DNA into a loop. This is in accordance with the facts that octamer-mediated looping has been established to be biologically relevant[7] and that the bacterial DNA has in vivo been shown to be highly condensed even in the absence of nonspecifically bound CIs to help facilitate the DNA looping.[16] This value of ΔG_{oct} is also in agreement with in vivo observations.[7, 16] ΔG_{tetr} differs somewhat from values estimated in vivo, however in vitro experiments lack parts of the machinery of a living cell and hence some processes may be more favorable in vivo, thus displaying a higher free energy. In comparison to the parameters describing the linear assay,[9] we modeled the observed increased cooperativity by lowering the intrinsic binding energies between CI and each operator site by 1.5 kcal/mol, thus making multiple CI binding energetically more favorable than monomeric CI binding. In total, with these parameters we thermodynamically allowed the biologically relevant octamer-mediated loop to be formed and the negative total free energy change associated with DNA looping, i.e., the sum of the two cooperative terms, indicated that looping on supercoiled DNA is thermodynamically favorable.

Supercoiling Tunes λ Immunity

Most interestingly, the minimum number of CI molecules in the cell necessary to form the pRM-repressible state (12 CI molecules) corresponds to a cellular concentration of 20 nM. Our results on the looping probability of supercoiled DNA (**Fig. 2D**) show that this minimum cellular concentration corresponds to looping the DNA between the operators 58% of time. Below 20 nM of CI, the probability of the operators being in the looped state drops rapidly. Thus, during induction by the SOS pathway, pRM remains in the repressible state until all of the free CI has been degraded with activated RecA. We propose that below this minimum threshold, the CIs abruptly vacate OR and OL and lytic repression collapses. Despite the low copy number of CIs in the cell, the pRM repressible state is robust to both perturbations in gene expression that can vary dramatically from cell to cell, and to CI nonspecific DNA binding that would otherwise lead to spontaneous transition to lytic development. Based on our findings we propose that supercoiling plays a role in stabilizing the pRM-repressible state such that CI synthesis from pRM does not impede induction.

Concluding Remarks

By using PNA as handles, we tethered supercoiled DNA plasmids and utilized

the assay for a single molecule study of the λ switch in a naturally supercoiled system. This assay has potential to examine how DNA in its natural supercoiled state interacts with proteins, enzymes, histones in nucleosomes, and the transcription and replication machinery. Interestingly, our results show that the λ switch is finely tuned to have an optimal, fast and efficient response to the cellular SOS system exactly at the cellular concentration corresponding to the minimum number of CI molecules capable of maintaining the CI repressible state.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

We acknowledge financial support from the University of Copenhagen excellence program.

References

1. Ptashne M. A Genetic Switch: Lambda Phage Revisited. Cold Spring Harbor, NY: Cold Spring Harbor Lab Press, 2004
2. Brooks K, Clark AJ. Behavior of lambda bacteriophage in a recombination deficient strain of Escherichia coli. J Virol 1967; 1:283-93; PMID:4918235
3. Baek K, Svenningsen S, Eisen H, Sneppen K, Brown S. Single-cell analysis of lambda immunity regulation. J Mol Biol 2003; 334:363-72; PMID:14623180; http://dx.doi.org/10.1016/j.jmb.2003.09.037
4. Little JW, Michalowski CB. Stability and instability in the lysogenic state of phage lambda. J Bacteriol 2010; 192:6064-76; PMID:20870769; http://dx.doi.org/10.1128/JB.00726-10
5. Bailone A, Levine A, Devoret R. Inactivation of prophage lambda repressor in vivo. J Mol Biol 1979; 131:553-72; PMID:159955; http://dx.doi.org/10.1016/0022-2836(79)90007-X
6. Meyer BJ, Ptashne M. Gene regulation at the right operator (OR) of bacteriophage lambda. III. lambda repressor directly activates gene transcription. J Mol Biol 1980; 139:195-205; PMID:6447796; http://dx.doi.org/10.1016/0022-2836(80)90304-6
7. Dodd IB, Shearwin KE, Perkins AJ, Burr T, Hochschild A, Egan JB. Cooperativity in long-range gene regulation by the lambda CI repressor. Genes Dev 2004; 18:344-54; PMID:14871931; http://dx.doi.org/10.1101/gad.1167904
8. Lewis D, Le P, Zurla C, Finzi L, Adhya S. Multilevel autoregulation of λ repressor protein CI by DNA looping in vitro. Proc Natl Acad Sci U S A 2011; 108:14807-12; PMID:21873207; http://dx.doi.org/10.1073/pnas.1111221108
9. Zurla C, Manzo C, Dunlap D, Lewis DE, Adhya S, Finzi L. Direct demonstration and quantification of long-range DNA looping by the lambda bacteriophage repressor. Nucleic Acids Res 2009; 37:2789-95; PMID:19276206; http://dx.doi.org/10.1093/nar/gkp134
10. Norregaard K, Andersson M, Sneppen K, Nielsen PE, Brown S, Oddershede LB. DNA supercoiling enhances cooperativity and efficiency of an epigenetic switch. Proc Natl Acad Sci U S A 2013; 110:17386-91; PMID:24101469; http://dx.doi.org/10.1073/pnas.1215907110
11. Lia G, Semsey S, Lewis DE, Adhya S, Bensimon D, Dunlap D, Finzi L. The antiparallel loops in gal DNA. Nucleic Acids Res 2008; 36:4204-10; PMID:18573800; http://dx.doi.org/10.1093/nar/gkn389
12. Normanno D, Vanzi F, Pavone FS. Single-molecule manipulation reveals supercoiling-dependent modulation of lac repressor-mediated DNA looping. Nucleic Acids Res 2008; 36:2505-13; PMID:18310101; http://dx.doi.org/10.1093/nar/gkn071
13. Lia G, Bensimon D, Croquette V, Allemand JF, Dunlap D, Lewis DE, Adhya S, Finzi L. Supercoiling and denaturation in Gal repressor/heat unstable nucleoid protein (HU)-mediated DNA looping. Proc Natl Acad Sci U S A 2003; 100:11373-7; PMID:14500788; http://dx.doi.org/10.1073/pnas.2034851100
14. Nelson PC, Zurla C, Brogioli D, Beausang JF, Finzi L, Dunlap D. Tethered particle motion as a diagnostic of DNA tether length. J Phys Chem B 2006; 110:17260-7; PMID:16928025; http://dx.doi.org/10.1021/jp0630673
15. Reichardt L, Kaiser AD. Control of lambda repressor synthesis. Proc Natl Acad Sci U S A 1971; 68:2185-9; PMID:4943790; http://dx.doi.org/10.1073/pnas.68.9.2185
16. Hensel Z, Weng X, Lagda AC, Xiao J. Transcription-factor-mediated DNA looping probed by high-resolution, single-molecule imaging in live E. coli cells. PLoS Biol 2013; 11:e1001591; PMID:23853547; http://dx.doi.org/10.1371/journal.pbio.1001591
17. Polikanov YS, Bondarenko VA, Tchernaenko V, Jiang YI, Lutter LC, Vologodskii A, Studitsky VM. Probability of the site juxtaposition determines the rate of protein-mediated DNA looping. Biophys J 2007; 93:2726-31; PMID:17573434; http://dx.doi.org/10.1529/biophysj.107.111245
18. Liu Y, Bondarenko V, Ninfa A, Studitsky VM. DNA supercoiling allows enhancer action over a large distance. Proc Natl Acad Sci U S A 2001; 98:14883-8; PMID:11742093; http://dx.doi.org/10.1073/pnas.261477898
19. Liu LF, Wang JC. Supercoiling of the DNA template during transcription. Proc Natl Acad Sci U S A 1987; 84:7024-7; PMID:2823250; http://dx.doi.org/10.1073/pnas.84.20.7024
20. DiNardo S, Voelkel KA, Sternglanz R, Reynolds AE, Wright A. Escherichia coli DNA topoisomerase I mutants have compensatory mutations in DNA gyrase genes. Cell 1982; 31:43-51; PMID:6297752; http://dx.doi.org/10.1016/0092-8674(82)90403-2
21. Anderson LM, Yang H. DNA looping can enhance lysogenic CI transcription in phage lambda. Proc Natl Acad Sci U S A 2008; 105:5827-32; PMID:18391225; http://dx.doi.org/10.1073/pnas.0705570105
22. Ackers GK, Johnson AD, Shea MA. Quantitative model for gene regulation by lambda phage repressor. Proc Natl Acad Sci U S A 1982; 79:1129-33; PMID:6461856; http://dx.doi.org/10.1073/pnas.79.4.1129