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Direct observation of a 91 bp LacI-mediated, negatively supercoiled DNA loop by atomic force microscope

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Abstract

*Escherichia coli* lactose repressor (LacI), a tetrameric protein, is a paradigmatic transcriptional factor that controls the expression of *lacZYA* in the *lac* operon. It specifically binds to the *O1, O2,* and *O3* operators of the *lac* promoter, forms DNA loops, and regulates transcription of the *lac* operon. In this article, utilizing combined techniques of DNA-nicking assay and AFM imaging, we directly observed a 91 bp LacI-mediated, negatively supercoiled DNA loop mimicking the DNA loop between the *O1 and O3* operators in the *lac* promoter.

Keywords

the *lac* repressor (LacI); DNA loop; atomic force microscope (AFM); the *lac* promoter; and the *lac* operon

The *E. coli* *lac* operon is a paradigmatic example of transcription regulation in bacteria [1,2]. This operon contains a *lac* promoter controlling the expression of three genes, *lacZ, Y,* and *A* [1,2]. The *lac* promoter has three specific operators *O1, O2* and *O3* with high affinity for a tetrameric protein, the *lac* repressor (LacI) [3]. Occupancy of these sites inhibits transcription from the *lac* promoter (Fig. 1A). Inducers, such as allolactose or IPTG, bind to LacI and allosterically inhibit its binding to the *lac* operators to allow transcription and expression of *lacZ, Y,* and *A* [3]. As a result *E. coli* cells are able to metabolize lactose [1,2]. The *lac O1, O2,* and *O3* are centered at 11, 412, and -82 bp of the promoter region respectively [4]. To increase the efficiency of repression [4,7] with no more than ten copies of LacI tetramers simultaneously present in each *E. coli* cell [5], the two auxiliary *O2* and *O3* operators increase the local concentration of LacI in the proximity of the *lac* promoter [6] by forming a LacI-mediated DNA loop with the primary *O1* operator.

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Previous studies demonstrated that LacI was able to form DNA loops between \( O1 \) and \( O2 \) or \( O1 \) and \( O3 \) \textit{in vitro} and \textit{in vivo} [8–12]. For instance, Borowiec et al. showed that LacI tetramer formed a 93 bp DNA loop between \( O1 \) and \( O3 \) using chemical footprinting assays [8]. Kramer et al. utilized gel electrophoresis and electron microscopy demonstrating that LacI was able to form small DNA loops between two symmetric operators \((O_{sym})\) [9,10]. Interestingly, the repression of \textit{lac} promoter is significantly enhanced if an auxiliary operator exists either upstream or downstream from the \( O1 \) operator [11] and the repression levels alternated between local maxima and minima [11,13–16]. The repression maxima are likely due to a stable DNA loop between the two operators when they face the same side of the DNA double helix [11]. An assay to detect such a LacI-mediated DNA loop would directly support this explanation. Here we report images of a 91 bp LacI-mediated DNA loop by atomic force microscopy (AFM) and show that it is negatively supercoiled.

**Materials and Methods**

**Proteins, chemicals, and reagents**

\textit{E. coli} LacI was purified by the method of Chen and Matthews [17] (\textit{E. coli} strains containing the plasmid overexpressing LacI was kindly provided by K. S. Matthews at Rice University). Restriction enzymes Nt.BbvCI and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA). All synthetic oligonucleotides were purchased from MWG-Biotech, Inc. (Huntsville, AL).

**Plasmid DNA templates**

Plasmid pOsOs91 was derived from plasmid pYZX43F [18] and constructed in two steps. The first step is to make a 140 bp DNA fragment carrying two \( O_{sym} \) operators and an Nt.BbvCI recognition site using two synthetic oligonucleotides FL723 \((5'-\text{TGTGGATCCCAGTGTAATTTGTGAGCGCTCACAATTAATGTGAGTTAGCTCACTCA TTAGGCACCCCCAGCTGAGGACTTTA-3'})\) and FL724 \((5'-\text{CATGAGGTACCAATTGTGAGCGCTCACAATTCCACACAACATACGAGCCG GAAGCATAAAGTCCAGCTCGGTTGTGGTG-3'})\) by polymerase chain reaction (PCR). This PCR DNA fragment was then inserted between the BamHI and KpnI sites of pYZX43F to yield pOsOs91. The center-to-center distance between the two \( O_{sym} \) operators is 91 bp mimicking the O1 and O3 operators in the \textit{lac} promoter.

**The DNA-nicking method**

The DNA-nicking method was described previously [19] with some modifications. Briefly, a typical DNA-nicking reaction mixture (320 \( \mu \)L) contained 20 mM Tris-acetate (pH 7.9 at 25 \( ^\circ \)C), 10 mM magnesium acetate, 1 mM DTT, negatively supercoiled plasmid pOsOs91, and LacI. All components were assembled on ice and incubated for 30 min at 37 \( ^\circ \)C. After the incubation, the supercoiled DNA templates were nicked by Nt.BbvCI at 37 \( ^\circ \)C for various times. Then, a large excess of a double-stranded oligonucleotide containing Nt.BbvCI recognition site were added to the reaction mixtures to inhibit the restriction enzyme activities. The nicked DNA templates were ligated by T4 DNA ligase in the presence of 1 mM of ATP at 37 \( ^\circ \)C for 5 min and the reactions were terminated by extraction with an equal volume of phenol. The DNA samples were precipitated with
ethanol and dissolved in 25 μL of 10 mM Tris-HCl buffer (pH 8.5). The linking number of the ligated DNA products was determined with 1% agarose gel electrophoresis in the absence or presence of 0.5 μg/ml of chloroquine and calculated from the gel images stained with SYBR Gold using KODAK 1D Image Analysis Software.

Atomic Force Microscopy

The LacI-pOsOs91 samples were prepared according to the DNA-nicking method as described above in the absence of Nt.BbvCI. After incubation at 37 °C for 30 min, the LacI-DNA complexes (10 μL) were deposited on a poly-L-ornithine-coated mica surface and incubated for 2 min at room temp. The droplet was rinsed away with 0.4 mL HPLC water and dried gently with compressed air. Images were acquired with a NanoScope IIIA MultiMode AFM microscope (Bruker, Santa Barbara, CA) operated in tapping mode using a 50–60 mV oscillation amplitude of uncoated, etched silicon tips with a resonance frequency of 75 kHz (NSC18, MirkoMasch, San Jose, CA) [20]. Areas of 1×1 μm² were scanned at a rate of 1.2 Hz and a resolution of 512×512 pixels. The DNA contour lengths were estimated by using Image Analysis Software ImageJ. The LacI-mediated 91 bp DNA loops appear as small protrusions from the round LacI molecules instead of distinctly visible, looped filaments as shown in our previously published work [19].

Molecular modeling

The DNA-loop was created with the GraphiteLifeExplorer modeling tool [21]. VMD software [22] was used to patch and render the DNA-loop structure with the LacI-DNA complex (PDB id 1Z04), which was originally modeled by patching several NMR-structures of fragments of LacI and DNA [23]. One (−) DNA supercoil was introduced to the DNA loop to match our experimental results for the LacI-mediated 91 bp DNA loop.

Results and Discussion

Fig. 1 shows our experimental design. First, we constructed plasmid pOsOs91 that carries two $O_{sym}$ operators. The center-to-center distance between the two $O_{sym}$ operators is 91 bp mimicking the $O1$ and $O3$ operators in the lac promoter. We also placed a nicking endonuclease Nt.BbvCI recognition site between the two $O_{sym}$ operators of the 91 bp region (Fig. 1). We used two different approaches to detect the 91 bp LacI-mediated, negatively supercoiled DNA loop. Our first approach was the DNA nicking assay in which Nt.BbvCI was used to nick pOsOs91 in the presence of LacI [19]. Fig. 2 shows our results. Binding LacI to the two $O_{sym}$ operators of pOsOs91 partially inhibited the nicking activities of Nt.BbvCI (Fig. 2A). Consistent with our previous results [19], binding of LacI to the two $O_{sym}$ operators established a topological barrier and divided the supercoiled DNA molecule into two topological domains, a 91 bp relaxed domain and a 4.2 kb supercoiled domain [19]. Our control experiments showed that in the absence of LacI the nicking enzyme Nt.BbvCI was able to completely nick supercoiled plasmid DNA within 30 seconds (Fig. 3A) and IPTG was able to inhibit the formation of the LacI-mediated topological barrier (Fig. 3B).

Using DNA nicking assays, we determined the stability of LacI-mediated topological barrier for pOsOs91 with a $t_{1/2}$ of 23.6±3.5 min (Fig. 2B and C). As expected, supercoils, 1.3±0.3, were constrained in the 91 bp DNA loop (Fig. 2D).
Next, we utilized atomic force microscope (AFM) to examine how one molecule of LacI divided a supercoiled DNA molecule into two topologically constrained DNA loops. We used supercoiled plasmid pOsOs91 with a supercoiling density of \(-0.06\) for our AFM studies. After LacI was added into solution mixtures, the LacI-plasmid complexes were deposited on a freshly cleaved mica surface and imaged. Fig. 4 shows our results. From these AFM images, we noticed a unique structure for \(\sim80\%\) of the supercoiled DNA molecule, a small protrusion from thicker, round particles bound to DNA (arrows of Fig. 4). The sizes of the particles were similar to that of LacI in our previous publication [19] with an average width or diameter of 13.8\(\pm1.4\) nm. This is also similar to the size of LacI in the crystal structure ([3]; Fig. 6). The average distance between the apex of the protrusion and the border of the particle was determined to be 17.5\(\pm2.3\) nm. We believe that this protrusion represents the 91 bp LacI-mediated, negatively supercoiled DNA loop. This result also suggests that the looped DNA is sharply bent. In contrast, in the absence of LacI, supercoiled DNA molecules imaged by AFM do not contain this kind of structures (Fig. 5).

We quantified the contour length of the DNA molecule and the 91 bp DNA loop. The average contour length of the DNA molecules was measured to be 1,475.8\(\pm155.4\) nm (Table 1). For B-form DNA with 0.34 nm per bp, this length was calculated to be 4,340\(\pm457\) bp, which matches the plasmid sequence length of 4,263 bp. The 91 bp DNA loop was determined to be 32.79\(\pm3.41\) nm or 96\(\pm10\) bp (Table 1).

Fig. 6 shows a molecular model of the physical interactions between LacI and \(lac\ O_{sym}\) operators that form a 91 bp DNA loop with one constrained (\(-\)) supercoil. The dimensions of the molecular model are comparable to the dimensions of the LacI-DNA complex with the 91 bp DNA loop. The model also shows a sharply folded structure for the 91 bp DNA loop with a sharp bend at the apex of the loop, which is consistent with the AFM images (Fig. 4). It is likely that the energetic cost of the sharp DNA bend in the 91 bp loop is compensated by the DNA-binding energy of LacI binding to the \(lac\ O_{sym}\) operators and (\(-\)) superhelicity in the DNA molecule.

Visualization of this loop is the first direct proof that a stable 91 bp supercoiled DNA loop can be formed upon binding of LacI to two \(lac\) operators. In bacteria, DNA is negatively supercoiled. It is likely that these AFM images reflect the real DNA looping structure \textit{in vivo}. Our results are consistent with recent results by Maher’s group that protein-mediated DNA looping in competition with RNA polymerase and transcription factors can be a major mechanism to repress transcription by bacterial RNA polymerase [16]. Negative supercoiling and HU protein greatly enhance the looping possibility between the O1 and O3 operators.

In summary, we detected the supercoiling within and directly observed a 91 bp LacI-mediated, negatively supercoiled DNA loop through a combination of a protein-pinched plasmid nicking assay and atomic force microscopy.

**Acknowledgments**

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References


Highlights

• Binding of LacI to the two $O_{sym}$ operators established a stable topological barrier;
• The 91 bp LacI-mediated, (−) supercoiled DNA loop was observed by AFM;
• A molecular model of the LacI-mediated, (−) supercoiled DNA loop was constructed.
Figure 1.
A strategy to study the LacI-mediated 91 bp supercoiled DNA loop. (A) A schematic illustration of the lac promoter region including the position of lac O1, O2, and O3. P_{lac} represents the lac promoter. (B) The map of plasmid pOsOs91. Plasmid pOsOs91 carrying two O_{sym} operators (closed rectangles) and an Nt.BbvCI recognition site is shown. (C) A schematic of the experimental procedure.
Figure 2.
Examining LacI-mediated 91 bp supercoiled DNA loop by the protein-pinched nicking assay. The nicking assays were performed as described under Material and Methods. Each reaction mixture (320 μL) contained 0.156 nM of pOsOs91, 2.5 nM of LacI, and 16 units of Nt.BbvCI. The reactions were incubated at 37 °C for the time indicated. Then a large excess of a double-stranded oligonucleotide containing an Nt.BbvCI recognition site was added to the reaction mixture to inhibit the restriction enzyme activities. The nicked DNA templates were ligated by T4 DNA ligase in the presence of 1 mM of ATP at 37 °C for 5 min and the reactions were terminated by phenol extraction. The DNA molecules were isolated and subjected to agarose gel electrophoresis in the absence (B) or presence (D) of 0.5 μg/mL of chloroquine. (A) The DNA nicking assay in the absence of T4 DNA ligase. (C) Quantification analysis of the time course. The percentage of supercoiled DNA was plotted against the reaction time. The curve was generated by fitting the data to a 1st-order rate equation to yield a t_{1/2} of 23.6±3.5 min.
Control experiments demonstrate that Nt.BbvCI rapidly nicked the supercoiled plasmid. (A) Protein-pinched nicking assays were performed as described under Materials and Methods. Each reaction mixture (320 μL) contained 0.156 nM supercoiled plasmid DNA template and 16 units of Nt.BbvCI. The reactions were incubated at 37 °C for the time indicated. Then a large excess of a double-stranded oligonucleotide contain an Nt.BbvCI recognition site was added to the reaction mixture to inhibit the restriction enzyme activities. The nicked DNA templates were ligated by T4 DNA ligase in the presence of 1 mM of ATP at 37 °C for 5 min and the reactions were terminated by phenol extraction. The DNA molecules were isolated and subjected to agarose gel electrophoresis. (B) DNA-nicking assays in the presence or absence of IPTG were performed as described in Materials and Methods and also in Fig. 3A.
Figure 4.
AFM images demonstrate the formation of the LacI-mediated, 91 bp supercoiled DNA loop. The AFM imaging experiments were performed as described under Materials and Methods.
Figure 5.
AFM images of supercoiled plasmids in the absence of LacI. The AFM imaging experiments were performed as described under Materials and Methods.
Figure 6.
LacI tetramer simultaneously binds to $O_{sym}$ operators and forms a 91 bp supercoiled DNA loop. This molecular model has been constructed as described under “Materials and Methods.”
Table 1
DNA contour lengths of pOsOs91 in the presence or absence of LacI

<table>
<thead>
<tr>
<th>DNA domain</th>
<th>Measured DNA contour length</th>
<th>DNA sequence length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nm</td>
<td>bp</td>
</tr>
<tr>
<td>Full length</td>
<td>1475.8±155.4</td>
<td>4340±457</td>
</tr>
<tr>
<td>91 bp DNA loop</td>
<td>32.79±3.41</td>
<td>96±10</td>
</tr>
</tbody>
</table>

*The measured DNA contour lengths in bp were calculated with the assumption of the standard B-form DNA for the plasmid; i.e., using a rise of 0.34 nm per base pair. 32 DNA molecules were used to measure the DNA contour length.*