Inhibition of the gyrA promoter by transcription-coupled DNA supercoiling in *Escherichia coli*

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The *E. coli* gyrA promoter (P gyrA) is a DNA supercoiling sensitive promoter, stimulated by relaxation of DNA templates, and inhibited by (−) DNA supercoiling in bacteria. However, whether P gyrA can be inhibited by transient and localized transcription-coupled DNA supercoiling (TCDS) has not been fully examined. In this paper, using different DNA templates including the *E. coli* chromosome, we show that transient and localized TCDS strongly inhibits P gyrA in *E. coli*. This result can be explained by a twin-supercoiled domain model of transcription in which (−) and (+) supercoiled domains are generated around the transcribing RNA polymerase. We also find that fluoroquinolones, such as ciprofloxacin, can substantially increase the expression of the firefly luciferase under the control of the P gyrA coupled to a divergent IPTG-inducible promoter in the presence of IPTG. This stimulation of P gyrA by fluoroquinolones can be also explained by the twin-supercoiled domain model of transcription. This unique property of TCDS may be configured into a high throughput-screening (HTS) assay to identify antimicrobial compounds targeting bacterial DNA gyrase.

DNA supercoiling plays a critical role in several crucial DNA transactions including DNA replication, recombination, transcription, and DNA repair. In bacteria, DNA molecules are usually (−) supercoiled. DNA supercoiling in *in vivo* is determined by counteractions of DNA topoisomerase I & IV (relaxation) and DNA gyrase (−) supercoiling. Inhibition of DNA gyrase activities by gyrase inhibitors causes the relaxation of the DNA templates or accumulation of (+) supercoiled plasmids and also induces the expression of DNA gyrase in bacteria. Deletion of *topA* from the chromosome results in the production of hypernegatively supercoiled DNA molecules at the exponential phase of bacteria. Recent genomic studies also showed that DNA supercoiling is critical for transcription regulation of many genes during bacterial cell growth.

Transcription can also disrupt localized DNA supercoiling in *vitro* and *in vivo*. Liu and Wang formulated a twin supercoiled domain model of transcription to explain how transcription affects localized DNA supercoiling. As the length of the RNA transcript increases, it becomes more and more difficult for the RNA-RNA polymerase complex to rotate around the DNA molecule. At a turning point, energetically, it is more practical to rotate the DNA about its own helical axis. Further translocation of the RNA-RNA polymerase along the DNA template generates a positively supercoiled domain in front of the transcribing RNA polymerase and a negatively supercoiled domain behind it. Many *in vitro* and *in vivo* results support this twin supercoiled domain model. For instance, in defined protein systems, transcription is able to drive close circular DNA templates to hypernegatively supercoiled status in the presence of DNA gyrase because DNA gyrase converts a fraction of the transient (+) supercoils into permanent (−) supercoils. Likewise, in *E. coli* topA strains, transcription at the exponential phase is able to drive close circular DNA templates to hypernegatively supercoiled because DNA gyrase converts (−) supercoiled domain into (+) supercoils.

Transcription-coupled DNA supercoiling (TCDS) is also able to activate supercoiling-sensitive promoters in bacteria. The best-studied case is the activation of bacterial Leu-500 promoter (P Leu-500) by TCDS, a promoter containing a single A-to-G mutation in the promoter region of the leu operon. Previous studies demonstrated that transcription-driven localized supercoiling rather than global supercoiling density was responsible for the activation of P Leu-500. The orientation of TCDS had opposite effects where (−) supercoiling domain activated P Leu-500 and (+) supercoiling domain suppressed the promoter. In our previously published studies, using uniquely designed linear plasmids, we demonstrated that transient and localized (−) DNA supercoiling...
can strongly activate P_{leu-500}. The activation of P_{leu-500} is dependent of the promoter strength and the length of RNA transcripts, unique properties of TCDS as predicted by the twin-supercoiled domain mechanism. We also demonstrated that TCDS could be generated on topologically open DNA molecules in E. coli cells. These results suggest that topological boundaries or barriers are not necessary for the generation of TCDS in vivo.

The E. coli gyrA promoter (P_{gyrA}) is another supercoiling sensitive promoter and stimulated by relaxation of DNA templates. Early mutation studies showed that the stimulation of P_{gyrA} stems from a 20 bp DNA sequence around the −10 region of P_{gyrA}. Since this 20 bp DNA sequence is intrinsically bent or curved, it is possible that the DNA bend or curvature functions as a supercoiling sensor for the activation by DNA relaxation. Nevertheless, whether P_{gyrA} can be inhibited by TCDS has not been examined. Here, using different DNA templates including the E. coli chromosome, we show that transient and localized (−) TCDS is able to strongly inhibit P_{gyrA} in E. coli.

Results and Discussion

In our previous studies, using an in vivo system that contains E. coli topA strain VS111(DE3)ΔlacZ or wild-type strain MG1655(DE3)ΔlacZ and a circular or linear plasmid DNA template, we demonstrated that transient and localized TCDS from a divergently-coupled transcription unit potently activated the supercoiling-sensitive promoter P_{leu-500}. In this study, we decided to utilize this system to examine whether and how TCDS inhibits a different supercoiling-sensitive promoter P_{gyrA}. For this purpose, we substituted P_{leu-500} with P_{gyrA}, divergently coupled to the strong IPTG-inducible promoter P_{T7A1/O4} (Fig. 1). The distance between these two promoters is 92 bp (Fig. 1A). As shown in Fig. 1C,D, we used 2 sets of 4 Rho-independent, rrnB T1 transcription terminators to block transcription from P_{T7A1/O4} and P_{gyrA} respectively. In this case, transcription is restricted to a selected region of the plasmids. Circular plasmid pZXD144 and linear plasmid pZXD150 were used to transform VS111(DE3)ΔlacZ or MG1655(DE3)ΔlacZ. After IPTG was added to E. coli cells in the early log phase, luciferase activities were used to determine the expression levels of the firefly luciferase controlled by P_{gyrA} coupled to a divergent IPTG-inducible promoter.

Figure 1. Experimental design of a pair of divergently coupled transcription units to examine transcription inhibition of P_{gyrA} by TCDS in vivo. (A) Divergently coupled promoters P_{T7A1/O4} and P_{gyrA}, respectively, control the expression of β-galactosidase (lacZ) and firefly luciferase (luc). (B) The DNA sequence of the pair of divergently coupled promoters, P_{T7A1/O4} and P_{gyrA}. Underlined are P_{gyrA} and P_{T7A1/O4} with −10 and −35 regions. (C,D) Maps of circular plasmid pZXD144 and linear plasmid pZXD150. Winged triangles represent Rho-independent rrnB T1 transcription terminators.
to monitor the inhibition of $P_{gyrA}$. Results in Fig. 2 show that TCDS strongly inhibits the supercoiling-sensitive $P_{gyrA}$ for both circular and linear plasmids. For example, TCDS from $E. coli$ RNA polymerase on pZXD144 inhibited 53% and 68% of $P_{gyrA}$ in VS111(DE3)$\Delta$lacZ and MG1655(DE3)$\Delta$lacZ, respectively, comparing with the activities of $P_{gyrA}$ in the absence of IPTG (Fig. 2B). TCDS on pZXD150 inhibited 42% and 63% of $P_{gyrA}$ in VS111(DE3)$\Delta$lacZ and MG1655(DE3)$\Delta$lacZ, respectively (Fig. 2D). Due to the fact that linear DNA templates cannot be permanently supercoiled \(^42\), these results unambiguously demonstrated that transient and localized TCDS, rather than global supercoiling, inhibits the divergently coupled $P_{gyrA}$. Interestingly, for circular plasmid pZXD144, the expression level of $\beta$-galactosidase is always higher in MG1655(DE3)$\Delta$lacZ than that in VS111(DE3)$\Delta$lacZ in the absence or presence of IPTG (Fig. 2A), which is consistent with our previously published results \(^43\). In contrast, for linear plasmid pZXD150, the expression level of $\beta$-galactosidase is lower in MG1655(DE3)$\Delta$lacZ comparing with that in VS111(DE3)$\Delta$lacZ (Fig. 2C). These results suggest that DNA supercoiling plays some roles in regulating the activities of $P_{gyrA}$ \(^39\). Please note that each $E. coli$ cell carries approximate 1 copy of a linear plasmid, the overall expression levels of firefly luciferase are much lower for linear plasmids \(^40\). Since the $topA$ strain VS111 is a DNA topoisomerase I deletion strain, it should have greater supercoiling fluctuations when disturbed by TCDS. As a result, $P_{gyrA}$ should be more sensitive to TCDS. Indeed, our results showed that $P_{gyrA}$ is more sensitive to the IPTG concentration, indicating that it is more sensitive to TCDS (Fig. 2B and D).

Figure 2. Inhibition of $P_{gyrA}$ by TCDS for circular plasmid pZXD144 (A, B) and linear plasmid pZXD150 (C, D). The activities of $\beta$-galactosidase (Miller's units) and firefly luciferase (RLU, relative light units) were determined as described under Methods and plotted versus the IPTG concentration. (A, B) $E. coli$ strains MG1655(DE3)$\Delta$lacZ (black squares and lines) and VS111(DE3)$\Delta$lacZ (red circles and lines) carrying pZXD144 were used. (C, D) $E. coli$ strains MG1655(DE3)$\Delta$lacZ (black squares and lines) and VS111(DE3)$\Delta$lacZ (red circles and lines) carrying pZXD150 were used. The standard deviation (SD) was determined according to results from three independent experiments.
Next, we examined how TCDS inhibits $P_{\text{gyrA}}$ on the *E. coli* chromosome. First, we placed a ~5 kb DNA fragment carrying the divergently coupled $P_{\text{gyrA}}$ and $P_{\text{TA1/O4}}$ promoters (Fig. 1A) into the attTn7 site of the *E. coli* chromosome (Fig. S1; the 84.2 min of the *E. coli* chromosome) using a procedure of transposon Tn7 to yield a wild-type strain FL1181 ($MG1655(\Delta E3)\Delta lacZattTn7-P_{\text{TA1/O4}}lacZ-P_{\text{gyrA}}\text{lacZ}$) and a topA strain FL1182 ($VS111(\Delta E3)\Delta lacZattTn7-P_{\text{TA1/O4}}lacZ-P_{\text{gyrA}}\text{lacZ}$). Due to technical difficulties, the four T1 transcription terminators were not included in these constructs. Similar to results for plasmid DNA templates as shown above, transcription by *E. coli* RNA polymerase can substantially inhibit transcription from $P_{\text{gyrA}}$ on the *E. coli* chromosome (Fig. 3A,B). For example, TCDS was able to inhibit 24% and 47% of $P_{\text{gyrA}}$ in FL1181 and FL1182, respectively. Interestingly, in the absence of IPTG, $P_{\text{gyrA}}$ in FL1182 is more active than that in FL1181 (Fig. 3B). As demonstrated previously, in the absence of IPTG, $P_{\text{TA1/O4}}$ is much more active in the wildtype strain MG1655 that is that in the topA strain VS111. Although the DNA templates may be more negatively supercoiled globally in VS111, the localized supercoiling around $P_{\text{gyrA}}$ in the wildtype strain MG1655 should be more negatively supercoiled than that in VS111 due to TCDS. In this way, the expression level of luciferase in VS111 should be higher than that in MG1655 in the absence of IPTG.

Since it was shown that gyrase inhibitors, such as coumermycin, quinolones, and novobiocin, are able to induce the expression of gyra and gyrB in bacteria, we also tested FL1181 and FL1182 with two gyrase inhibitors, novobiocin and ciprofloxacin, and examined whether these two gyrase inhibitors are able to increase the firefly lucerase expression under the control of $P_{\text{gyrA}}$. At the early exponential stage, novobiocin slightly enhanced the expression of firefly lucerase in FL1181 (Fig. 3C) and did not have much effect on the expression of firefly lucerase in the topA strain FL1182 (Fig. 3C). Ciprofloxacin at low concentrations slightly stimulated the expression of firefly lucerase for both strains (Fig. 3D; the differences appear to be statistically insignificant) and inhibited the expression of firefly lucerase in FL1181 at 50 μM (Fig. 3D). Intriguingly, in the presence of IPTG, the stimulation of firefly lucerase expression by ciprofloxacin was significantly amplified (Fig. 3F) although ciprofloxacin at high concentrations completely inhibited the expression of firefly lucerase in FL1181 and FL1182 (Fig. 3D). Intriguingly, in the presence of IPTG, novobiocin only inhibited DNA gyrase activities and does not form gyrase-novobiocin-DNA complexes, it should not significantly enhance or inhibit the expression of firefly lucerase in FL1181 and FL1182 (Fig. 4A and C). Other antibiotics, due to not affecting DNA supercoiling...
Figure 3. Strong inhibition of the supercoiling-sensitive P_{gyrA} by TCDS on the chromosome. (A,B) TCDS assays for P_{gyrA} on the chromosome. E. coli strains FL1181 (MG1655(DE3)ΔlacZ attTn7::PT7A1/O4lacZ-P_{gyrA}luc; black squares and lines) and FL1182 (VS111(DE3)ΔlacZ attTn7::PT7A1/O4lacZ-P_{gyrA}luc; red circles and lines) were used. The activities of β-galactosidase and firefly luciferase were determined as described under Methods and plotted versus the IPTG concentration. (C,D) Effects of novobiocin (C) and ciprofloxacin (D) on P_{gyrA} of FL1181 (black squares and lines) and FL1182 (red circles and lines) in the absence of IPTG. (E,F) DNA gyrase inhibitors significantly enhanced the expression of firefly luciferase for FL1181 and FL1182 in the presence of IPTG. Overnight cell cultures were diluted 100-fold and grown until OD600 reached ~0.2. Then 0.5 mM of IPTG and various concentrations of ciprofloxacin or other antibiotics were added to the cell cultures. After 30 min incubation, the activities of β-galactosidase and firefly luciferase were determined described under Methods. (C) Ciprofloxacin (CIPX) inhibited the expression of β-galactosidase. (D) CIPX greatly enhanced the expression of firefly luciferase. The standard deviation (SD) was determined according to results from three independent experiments.
status in vivo, should not be able to enhance the expression of firefly luciferase. In contrast, they inhibited the expression of firefly luciferase and β-glactosidase in FL1181 and FL1182.

Summary. Here, using a unique in vivo system, we demonstrated that transient and localized (−) TCDS provided by E. coli RNA polymerase could inhibit the P gyrA at the plasmid and chromosomal levels. We also found that fluoroquinolones, such as ciprofloxacin, were able to substantially increase the expression of the firefly luciferase under the control of the P gyrA in the presence of IPTG. This unique property of TCDS can be effectively used to screen and identify antimicrobial compounds targeting bacterial DNA gyrase.

Methods

Materials. Kanamycin, lysozyme, and ortho-Nitrophenyl-β-galactoside (ONPG) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Ampicillin and bovine serum albumin (BSA) were bought from Fisher Scientific (Fairlawn, NJ). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Antracite, Inc (Maumee, Ohio). All restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). Pfu DNA polymerase was obtained from Stratagene, Inc. (La Jolla, CA). All synthetic oligonucleotides were purchased from Eurofins Genomics (Huntsville, AL). Plasmid and DNA fragment cleaning kits including QIAprep Spin Miniprep Kit, QIAquick Gel Extraction Kit, and QIAquick Nucleotide Removal Kit were obtained from QIAGEN, Inc. (Valencia, CA). Luciferase Assay System was bought from Promega Corporation (Madison, WI).

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**Figure 4.** The stimulation of expression of firefly luciferase of FL1181 (A) and FL1182 (C) by fluoroquinolones in the presence of 0.5 mM IPTG. CIXP, LVF, EFX, NFX, and novobiocin represent ciprofloxacin, levofloxacin, enrofloxacin, norfloxacin, and novobiocin, respectively. Three bars from left to right represent luciferase activities in the presence of 0, 5, and 10 μM of fluoroquinolones, respectively. (B and D) The inhibition of expression of firefly luciferase by other antibiotics (none gyrase inhibitors) for FL1181 (B) and FL1182 (D). RMP, KM, AMP, and TC represent rifampicin, kanamycin, ampicillin, and tetracycline, respectively. The following are concentrations used in the experiments from left to right: AMP, 0, 150, 300 μM; KM, 0, 40, 80 μM; RMP, 0, 25, 50 μM; TC, 0, 10, 20 μM. The standard deviation (SD) was determined according to results from three independent experiments.
Plasmid DNA templates. Circular plasmid pZXD133, a derivative of pBR322, was described previously. Plasmid pZXD144 was constructed by inserting a 70 bp synthetic oligomer harboring a P_gyrA into the BamHI and HindIII sites of pZXD133. In this case, P_gyrA is divergently coupled to P_T7A1/O4 (Fig. 1). Linear plasmid pZXD150 was described previously.

Bacterial strains. E. coli strains MG1655 (DE3) and VS111 (DE3) were described previously. E. coli strains FL1181 (MG1655(DE3) ΔlacZ attTn7::P_T7A1/O4lacZ-P_gyrAluc) and FL1182 (VS111(DE3) ΔlacZ attTn7::P_T7A1/O4lacZ-P_gyrAluc) were created by utilizing a Tn7-based site-specific recombination system as follows. A 5.1 kb DNA fragment harboring the divergently coupled P_gyrA and P_T7A1/O4 promoters controlling the luc and lacZ genes, respectively, was inserted into the attTn7 site of the E. coli chromosome to yield FL1181 and FL1182 in which the IPTG-inducible P_T7A1/O4 controls the expression of β-galactosidase.

The expression of β-galactosidase. The expression level of β-galactosidase was measured as described in previous publications. Briefly, 100 mL of LB was inoculated with 1 mL of overnight bacterial cell culture at ratio of 1:100 until OD 600 = ~0.2. 100 μL of bacterial cell culture was added to 900 μL of Z-buffer (60 mM Na2HPO 4, 40 mM NaH 2PO 4, 10 mM KCl, 1 mM MgSO 4, and 50 mM β-mercaptoethanol). Then, 60 μL of chloroform and 30 μL of 0.1% SDS were added to lyse the cells. After cell lysates were incubated at 30 °C for 5 minutes, 200 μL of ONPG (4 mg/mL) was added. After another 15 min of incubation at 30 °C, 500 μL of 1 M Na 2CO 3 was added to stop the reaction. After cell debris was removed by centrifugation at 13,000 rpm for 1 min, the OD 420 and OD 550 values were measured in a Cary 50 spectrophotometer. β-Galactosidase activities (E) were calculated using equation:

$$ E = 1000 \times \frac{OD_{420} - 1.75 \times OD_{550}}{t \times v \times OD_{600}} $$

where t and v, respectively, represent reaction time and cell culture volume.

Luciferase assay. The expression of the firefly luciferase in E. coli were monitored by using the luciferase assay as described in our previous publication.

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Author Contributions
F.L. designed research; S.D., K.D., and X.Z. performed research; F.L. analyzed data; F.L. wrote the paper.

Additional Information
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