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Distribution of gyrase and topoisomerase IV on bacterial nucleoid: implications for nucleoid organization

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ABSTRACT

We explored the existence of nucleoid DNA loops in *Escherichia coli* by studying the distribution of bacterial type II topoisomerases (Topo IIs). Norfloxacin-induced high molecular weight (HMW) DNA fragmentation of nucleoid, an event reminiscent of the excision of eukaryotic chromosomal DNA loops mediated by topoisomerase II (TOP2). The size of the HMW DNA fragments induced by norfloxacin was affected by transcription, translation and growth phases of bacteria. The involvement of bacterial Topo IIs in the generation of these HMW DNA fragments is supported by the following observations: (i) the excised loop-sized DNA fragments were covalently linked to proteins; (ii) the norfloxacin-induced excision of DNA loops was highly reversible; (iii) coumermycin A1 antagonized the excision of DNA loops induced by norfloxacin; (iv) this antagonistic effect was reduced in either gyrase or *topo IV* mutants conferring coumarin resistance and (v) norfloxacin-induced reversible, gyrase-mediated DNA cleavage *in vitro*. Importantly, studies on coumarin- and/or quinolone-resistant mutant strains showed that DNA gyrase, rather than topoisomerase IV, plays the major role in the generation of loop-sized HMW DNA fragments. In sum, our study suggests a potential role of Topo IIs in the arrangement of DNA supercoiling loop domains in prokaryotic cells.

INTRODUCTION

Two levels of DNA organization in bacterial nucleoid and eukaryotic chromatin have been suggested by earlier physical and chemical studies, these being the supramolecular looped organization and short-range structure (1–12). The looped organization of bacterial nucleoid, which is suggested by the existence of supercoiling-independent looped domains, is presumably responsible for the long-range, higher-order architecture of nucleoid DNA (8,13,14). The short-range structure might be important in restraining DNA in a negative-supercoiled state (12–14). In eukaryotic cells, chromosome DNA is coated with at least an equal mass of proteins, forming a complex termed chromatin (1,12,15). Proteins, such as DNA topoisomerases, histones, histone-like proteins and chromatin-associated proteins, have been suggested to play important roles in both the regulation and maintenance of chromatin structure (1,9–12,15–20). Importantly, these proteins not only play roles in structuring chromatin, but also in regulating DNA metabolism, such as replication and transcription (1,9,21).

In the short-range nucleosome structure, DNA–histone complexes are the fundamental units of eukaryotic chromatin responsible for the first order of DNA packing. Each nucleosome associates with one histone H1 molecule and the bead-like structure coils into a 30 nm solenoid fiber. These fibers are then further folded into higher-order structures. A radial loop model has been proposed for the supramolecular organization of eukaryotic chromosome (15,20,22). In this model, chromosome DNA is organized into large loops (estimated as 50 to 100 kb in length) by periodic attachment to the high-salt-insoluble, protein-based nuclear matrix/scaffold (9,20,22–24). The matrix/scaffold-associated regions (M/SARs) are repeated *cis*-acting DNA elements forming the bases of looped domains (9,22–25). DNA TOP2 and other matrix-associated proteins have been suggested to interact with M/SAR sequences and form the protein complexes for loop anchorage sites (11,22,25,26). Eukaryotic TOP2-mediated excision of DNA loops has therefore been extensively used to study the organization of chromosomal long-range structure (11,17,27,28).

Several studies have revealed that many histone-like components, such as H-NS, HU and IHF, participate in the organization of short-range structure of bacterial nucleoid (8,12). Although the presence of long-range, constrained DNA supercoiling domains in prokaryotic nucleoid similar to those in eukaryotic cells has been suggested from a number of observations (3,6,13,29–31), our knowledge of the *cis*- and

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trans-components maintaining the supramolecular looped domains of bacterial nucleoid DNA and the factors that define the boundaries of domains is limited (3,6,13). The domain structure was apparent in early electron microscope (EM) images of nucleoids in disrupted *Escherichia coli* (6,30,31) and in later atomic force microscopy (AFM) images (14,32). It is important to note that, although the nature of the cellular components at the bases of the potential looped arrangements is not known, each DNA loop has been shown to be topologically independent (2,13). Subsequent studies, including measuring the output of supercoil-sensitive promoters, site-specific recombination between distinct chromosomal sites, and sequence-specific DNA localization, provided information on the dynamic and spatial aspects of the nucleoid looped organization (33–37). The involvement of bacterial topoisomerase IIs (Topo IIs) has also been suggested from density gradient studies showing that the quinolone antibiotic, oxolinic acid, causes cleavage of nucleoid DNA into large DNA fragments (38,39). However, the factors involved in the regulation of the long-range architecture of bacterial nucleoid remain largely unexplored.

Two type II topoisomerases, DNA gyrase and topoisomerase IV (Topo IV), have been identified, and act in concert with topoisomerase I (TopA), making an important contribution to the steady-state levels of supercoiling in *E.coli* (40). In addition, both Topo IIs have been found to be targets for many quinolone antibiotics (40–42). In mammalian cells, TOP2 excises chromosomal DNA loops (~50 to 100 kb) in cells treated with TOP2-targeting drugs (11,27,28). Here, we treated bacteria with a quinolone, norfloxacin, to induce DNA fragmentation of nucleoid DNA and examined the relative contribution of gyrase and Topo IV to norfloxacin-induced excision of high molecular weight (HMW) nucleoid DNA fragments. First, we showed that bacterial nucleoid DNA was rapidly cleaved into loop-sized DNA fragments (~50 to 100 kb) by norfloxacin treatment, indicating the potential existence of nucleoid DNA loops. We then examined whether this effect was mediated by bacterial Topo IIs. This was demonstrated to be the case by the tight association of proteins with HMW DNA fragments, the reversible nature of DNA loop excision, and the ability of coumermycin A1 to antagonize the fragmentation. We also determined that DNA gyrase was more active in the generation of loop-sized HMW DNA fragments than DNA Topo IV. In addition, studies using mutant strains suggested that *E.coli* TopA and structural maintenance of chromosome (SMC) proteins might also contribute to the overall organization of nucleoid DNA loops. Taken together, our data suggest the existence of Topo II-modulated supercoiling loop domains in higher-order nucleoid DNA organization in prokaryotic cells.

MATERIALS AND METHODS

Chemical, drugs and enzymes

Unless otherwise stated, all chemicals and drugs were purchased from Sigma Chemical Co. Proteinase K (PK) was obtained from Roche Applied Science Co. All drugs were dissolved in dimethyl sulfoxide (DMSO) and were stored in aliquots frozen at −20°C. Purified *E.coli* DNA gyrase was kindly provided by Dr Martin Gellert (National Institutes of Health, MD, USA).

Table 1. Bacterial strains used

Strain	Genotype	Reference
LZ35	LZ36, except *gyrA*^{L83}	(43)
LZ36	W3101λ, except *zei-723::* Tn10, *kan*^{R}	(43)
LZ37	LZ36, except *gyrA*^{L83} *parC*^{K84}	(43)
LZ38	LZ36, except *parC*^{K84}	(43)
DPB923	DPB924 *topA10*	(44)
DPB924	Wild-type	(44)
CC4207	DPB924 *topA10* Δ*mukB::kan*	(44)
CC4208	DPB924 Δ*mukB::kan*	(44)
RFM443	*rpsL galK2* Δ*lac74*	(56)
RFM445	REFM443 *gyrB221* (Cou^{R}) *gyrB203*(Ts)	(56)
1358	F^{-} *metB argE ilv tna*	(57)
1359	1358 *gyrB234* (Cou^{R}, Ts)	(57)
2822	1358 *parE*^{R132C}*:cat*	(57)
2824	1359 *parE*^{R132C}*:cat*	(57)

Bacteria strains and growth conditions

Bacterial strains, LZ35–38 (43), 1358, 1359, 2819, 2822 and 2824, were obtained from Dr Nicholas R. Cozzarelli (UC Berkeley, USA), strains DPB923, DPB924, CC4207 and CC4208 (44) from Dr Stuart Austin (National Cancer Institute, USA), and strains RFM443 and RFM445 from Dr Yuk-Ching Tse-Dinh (New York Medical College, NY). The genotypes of the *E.coli* strains used are described in Table 1. All bacterial strains were maintained in Luria–Bertani (LB) medium at 37°C with shaking (250 r.p.m.) unless otherwise indicated.

Encapsulation of cells and drug treatment

After 30 min of norfloxacin treatment at 37°C (dosage as indicated in the Figure legends), the cells were spun down and resuspended to an optical density at 595 nm of 1 in 50 μl of LB medium. After mixing with an equal volume of 1.0% (weight/volume) agarose premelted in LB, the samples were loaded into agarose plug makers (100 μl per plug). After solidification, the bacteria-containing agarose plug was placed in 400 μl of lysis buffer [50 mM Tris (pH 8.0), 10 mM EDTA and 0.5% SDS] with or without 0.5 mg/ml of PK and incubated at 55°C for 24 h. The integrity of the nucleoid DNA was then analyzed by pulsed-field gel electrophoresis (PFGE).

PFGE

PFGE was performed for 10 h at 14°C in 0.5× TBE [45 mM Tris-base, 45 mM boric acid, 2 mM EDTA (pH 7.6)] in a contour-clamped homogeneous electric field apparatus (CHEF, Bio-Rad) using conditions of 6 V/cm, an angle of 120°, and linear ramping with pulse times of 5–15 s and 15–60 s. After electrophoresis, the gel was stained with ethidium bromide for 30 min at room temperature, then photographed under ultraviolet (UV) light. For clarity, the colors in the PFGE figures were inverted using the Photoshop program. Fragment size was determined by comparison with a λ ladder PFG marker (New England Biolabs Inc.).

Gyrase-mediated DNA cleavage assays *in vitro*

Briefly, BamHI-digested YEpG DNA was labeled at the 3′ end using [α-^{32}P]dATP and the large fragment of *E.coli* DNA polymerase I. The reaction mixture (20 µl final volume) contained 50 mM Tris (pH 8.0), 100 mM KCl, 8 mM $MgCl_2$, 0.1 mM DTT, 0.5 mM EDTA, 30 µg/ml of BSA, ~10 ng of *E.coli* DNA gyrase and 10 ng of end-labeled [^{32}P] YEpG DNA. Norfloxacin was added at the indicated concentration to the reaction mixture, which was then incubated at 37°C for 30 min, and the reaction terminated by addition of 5 µl of stop buffer (5% SDS and 2.5 mg/ml of PK) and incubation for 1 h at 37°C. The reaction products were separated on a 0.8% agarose gel in 0.5× TPE buffer [45 mM Tris-phosphate (pH 8.0), 1 mM EDTA], then the gel was dried onto 3 MM chromatographic paper and autoradiographed at −80°C using Kodak XAR-5 films.

RESULTS

Norfloxacin induces DNA fragmentation of nucleoid

Bacterial cells (LZ36) were treated with norfloxacin, encapsulated in agarose plugs, and the production of HMW DNA fragments measured by PFGE. As shown in Figure 1 (the left panel), norfloxacin caused dose-dependent production of HMW fragments. At concentrations of norfloxacin >3 µM, nucleoid DNA was mainly cleaved into loop-sized HMW DNA fragments (~50–100 kb), similar in size to the average DNA length of excised chromosomal DNA loops induced by TOP2-tageting drugs in eukaryotic cells. This conserved phenomenon (i.e. excision of nucleoid DNA loops) strongly suggests that the bacterial chromosome is organized into supramolecular looped domains like those in eukaryotic cells. In addition, when norfloxacin was used at concentrations >3 µM, a small fraction of the nucleoid DNA was seen as more slowly migrating HMW DNA fragments (200 to 600 kb and higher, indicated as 'larger HMW DNA fragments' on Figure 1). Norfloxacin-induced similar HMW DNA fragmentation in different kinds of bacteria, including *Staphylococcus aureus, Streptococcus mutans, Klebsiella pneumoniae and Helicobacter pylori* (data not shown). Taken together, and consistent with a previous report using oxolinic acid and sedimentation analysis (38,39), our results demonstrated that another quinolone, norfloxacin, induced a similar cleavage pattern of bacterial nucleoid.

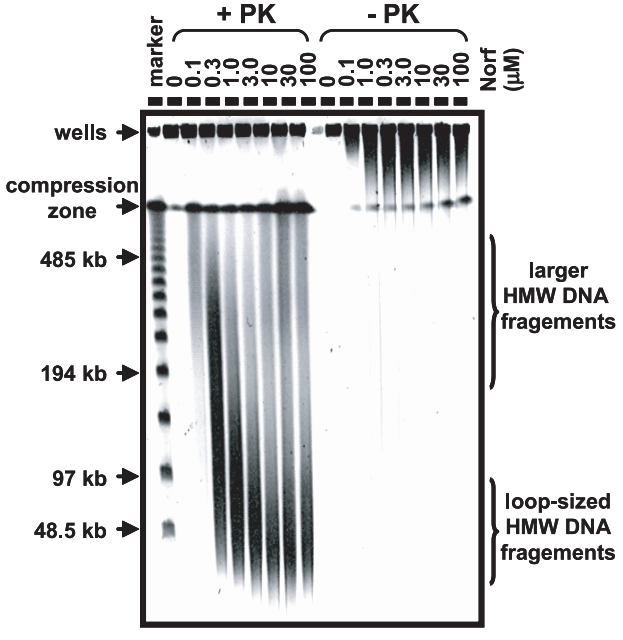

Figure 1. Rapid fragmentation of nucleoid DNA into protein-linked HMW DNA fragments in *E.coli* treated with norfloxacin. Bacterial cells were treated with 0.1–100 µM norfloxacin for 30 min at 37°C, then were encapsulated in agarose plugs and subjected to lysis with SDS in the presence or absence of PK. Nucleoid integrity was then analyzed by PFGE as described in the Materials and Methods. [PK: protease K; Norf: norfloxacin].

The norfloxacin-induced HMW DNA fragments of bacterial nucleoid are tightly linked to protein

We further characterized the HMW DNA fragments produced by treatment of bacterial cells with 10 µM norfloxacin. When drug-treated cells were lyzed in the absence of PK, the loop-sized DNA fragments generated by norfloxacin treatment disappeared completely and accumulation of larger HMW DNA fragments in the region between the compression zone and the wells was observed. It should be noted that the amount of these protein-linked HMW DNA fragments increased as the concentration of norfloxacin increased. Our results also suggested that both the loop-sized and larger HMW DNA fragments were tightly associated with proteins, since the mobility of the HMW DNA fragments generated by norfloxacin was significantly less when PK was omitted from the lysis buffer (Figure 1, right panel). This suggests the involvement of a DNA topoisomerase in this nucleoid DNA fragmentation process, since topoisomerase is known to be covalently linked to broken ends of DNA in the topoisomerase–DNA cleavable complex (39,45–48).

Modulation of norfloxacin-induced HMW DNA fragmentation by transcription or translation inhibitors

Previous reports have shown that many cellular processes can have different effects on the structure and membrane attachment of nucleoid in *E.coli*, especially transcription (49–51). In bacterial cells, translation is tightly coupled to transcription (52). We therefore examined the effect of inhibitors of transcription or translation on the norfloxacin-induced DNA fragmentation. Treatment of LZ36 cells with either translation or transcription inhibitors did not result in HMW DNA fragmentation (Figure 2A and B). Pretreatment of cells with the translation inhibitors, chloramphenicol, tetracycline and spectinomycin, had no effect on the norfloxacin-induced HMW DNA fragmentation of nucleoid DNA (Figure 2A and data not shown). However, pretreatment with 1 mM kanamycin caused a change in the pattern of norfloxacin-induced HMW DNA fragments (Figure 2A). Thus, depending on their mechanisms of action, translation inhibitors had different effects on the DNA cleavage pattern of nucleoid induced by norfloxacin treatment. As shown in Figure 2B, the transcription inhibitor, rifampicin, also had an effect on the HMW DNA fragmentation generated by norfloxacin,

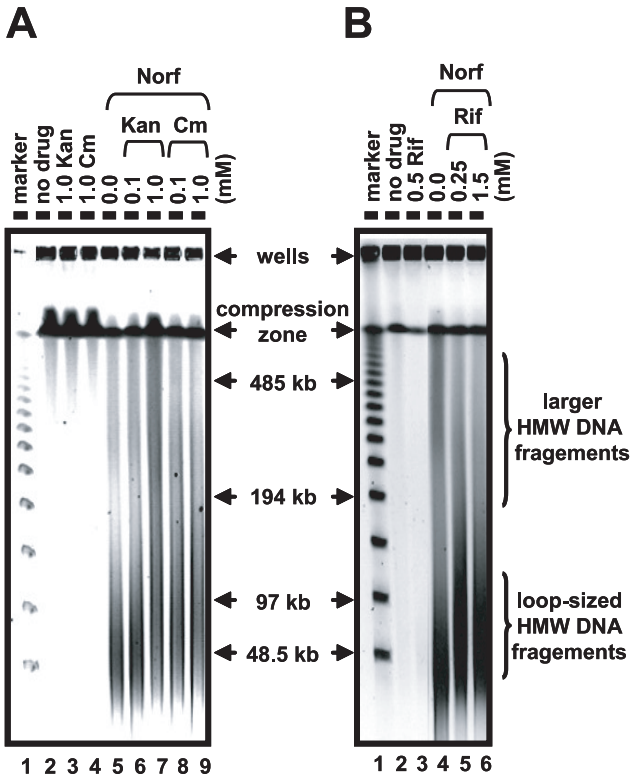

Figure 2. Translation (**A**) and transcription (**B**) processes modulate HMW DNA fragmentation in *E.coli* treated with norfloxacin. Bacterial cells were pretreated for 30 min at 37°C with the indicated concentration of inhibitors of translation (kanamycin or chloramphenicol) or transcription (rifampicin), then for another 30 min with 10 μM norfloxacin in the continued presence of the inhibitor. The cells were then encapsulated and PFGE performed to examine the integrity of genomic DNA as described in the Materials and Methods. [Kan: kanamycin; Cm: chloramphenicol; Rif: rifampicin].

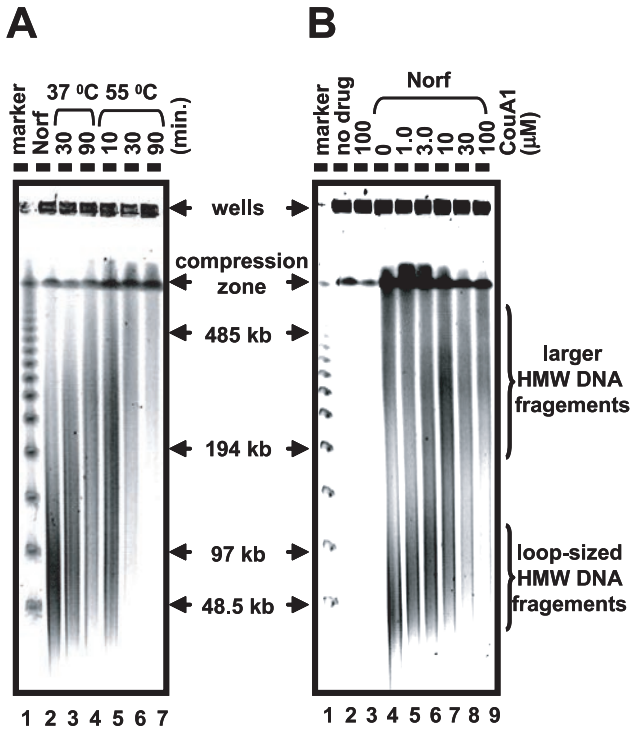

Figure 3. Norfloxacin-induced excision of DNA loops is mediated by DNA topoisomerases. (**A**) Reversibility of norfloxacin-induced HMW nucleoid DNA fragmentation. Cells were treated for 30 min at 37°C with 10 μM norfloxacin, washed and encapsulated in agarose plugs. The plugs were then subjected to different reversal conditions in drug-free LB media for various times before analysis of fragmentation. (**B**) Coumermycin A1, a bacterial TOP2 catalytic inhibitor, inhibits norfloxacin-induced HMW nucleoid DNA fragmentation. Experiments were performed as described in Figure 2, except that coumermycin A1 was used instead of translation or transcription inhibitors. [CouA1: coumermycin A1].

the center of the looped-sized DNA fragment band shifting from 50 kb to ~75 kb in the presence of rifampicin. Taken together, these results suggest that both transcription and a kanamycin-sensitive translation process are involved in norfloxacin-induced HMW DNA fragmentation, possibly through modulation of the distribution of cleavage sites on nucleoid DNA.

The norfloxacin-induced excision of nucleoid HMW DNA fragments is reversible and can be antagonized by coumermycin A1

The fact that the norfloxacin-induced nucleoid HMW DNA fragments were linked to proteins immediately suggests the involvement of a topoisomerase. The DNA fragmentation induced by Topo II-targeting drugs/poisons is due to the formation of reversible Topo II–DNA–drug cleavable complexes (27,39,46,53), and heat-induced reversal (i.e. 55°C treatment) is one of the unique properties of these complexes (39,53). To confirm the involvement of Topo II in the excision of nucleoid DNA loops, we therefore tested the antagonistic effect of a Topo II catalytic inhibitor and for heat reversibility.

When the norfloxacin-treated cells were heated at 55°C for various times prior to SDS lysis, the norfloxacin-induced HMW DNA fragments disappeared in a time-dependent manner, most being lost after 1.5 h at 55°C (Figure 3A, lanes 5–7). It should be noted that, although less effective, reversal by drug removal from the medium at 37°C also caused reversal of norfloxacin-induced HMW DNA fragmentation (Figure 3A, compare lane 2 to lanes 3 and 4).

DNA gyrase is the target of the coumarin group of antibacterial agents (54,55), which are known to bind specifically to the 24 kDa N-terminal subdomain of gyrase B, promote dimerization and thereby inhibit the ATPase activity of DNA gyrase (54). Interestingly, coumermycin A1 has been shown to have an antagonistic effect on cell killing induced by gyrase-targeting poisons, such as quinolone antibiotics (45,55). We therefore tested its effect on the norfloxacin-induced excision of nucleoid DNA loops. As shown in Figure 3B, coumermycin A1 did not induce HMW DNA fragmentation (lane 3), but blocked norfloxacin-induced DNA fragmentation in a dose-dependent manner (lanes 4–9). Pretreatment of cells with coumermycin A1 at concentrations >10 μM resulted in a marked reduction in norfloxacin-induced loop-sized fragmentation of nucleoid DNA, i.e. norfloxacin-induced nucleoid DNA fragmentation was effectively antagonized by coumermycin A1. Thus, the excision of nucleoid DNA loops following norfloxacin treatment was most likely mediated through a DNA Topo II.

The antagonistic effect of coumermycin A1 on norfloxacin-induced HMW DNA fragmentation is due to a specific inhibitory action on Topo II

DNA gyrase and Topo IV are targeted and inhibited by norfloxacin and coumermycin A1 (42,43,55). Mutant strains carrying coumarin-resistant gyrase and/or *topo IV* mutations (56,57) were used to confirm that the molecular targets of coumermycin A1 were DNA gyrase and Topo IV. For example, coumermycin A1 cannot inhibit the catalytic activity of coumarin-resistant gyrase and therefore, coumermycin A1 will not be able to antagonize mutant gyrase-mediated DNA fragmentation induced by norfloxacin in coumarin-resistant strains. As shown in Figure 4, the extent of norfloxacin-induced HMW DNA fragmentation was less in gyrase mutants (RFM445 and 1359) than in wild-type cells (RFM443 and 1358), an observation consistent with the temperature-sensitive phenotype of the gyrase mutations of the strains (Table 1). Coumermycin A1 antagonized norfloxacin-induced HMW DNA fragmentation in wild-type RFM443 and 1358 cells, but not in coumarin-resistant RFM445 cells [RFM443 *gyrB221* (Cou^R)] and 1359 [*gyrB234* (Cou^R)] cells. These results confirmed that DNA gyrase is an important *in vivo* target for both norfloxacin and coumermycin A1. A similar antagonistic effect of coumermycin on norfloxacin-induced HMW DNA fragmentation was seen using the *topo IV* mutant 2822 [1358 *parE132* (Cou^R)] (Figure 4B, compare lanes 9 and 10), but not using the double mutant 2824 [1358 *gyrB234, parE132* (Cou^R)] (Figure 4B, compare lanes 12 and 13). These results are consistent with the later observation (Figure 6B) that, following norfloxacin treatment, Topo IV caused less extensive cleavage of nucleoid DNA and at different sites, compared to gyrase.

Norfloxacin induces gyrase-mediated reversible DNA cleavage *in vitro*

We further tested the ability of norfloxacin to induce Topo II-mediated DNA cleavage *in vitro* using purified recombinant DNA gyrase as described previously (47,48). As shown in Figure 5A, norfloxacin-induced dose-dependent formation of DNA double-stranded breaks only in the presence of DNA gyrase (lanes 2–5, 7–10). In addition, the norfloxacin-induced, gyrase-mediated DNA cleavage was markedly stimulated (~100-fold) in the presence of 1 mM ATP (compare lanes 5 and 8). We also examined whether the norfloxacin-induced cleavage of DNA in the *in vitro* cleavage assay was reversible and demonstrated, for the first time, that gyrase-mediated cleavage of DNA was highly reversible

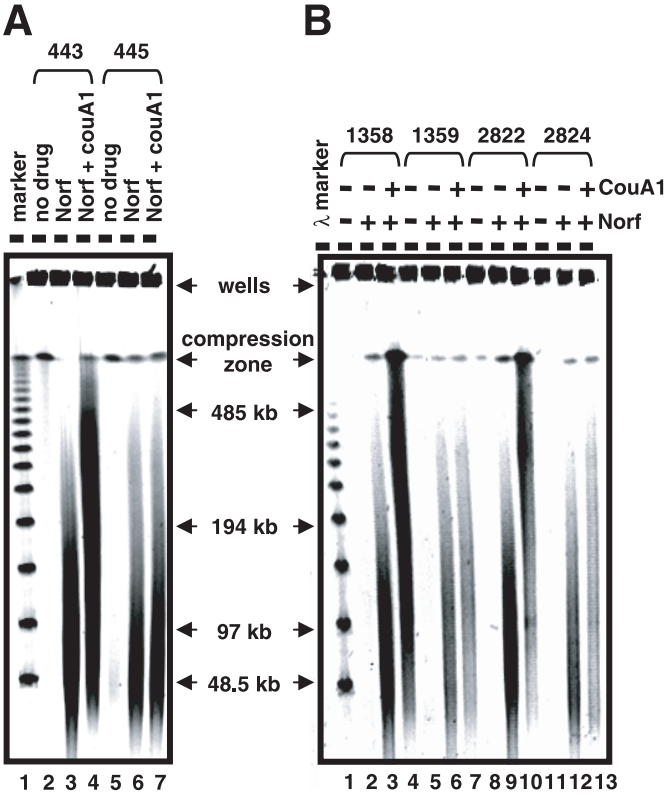

Figure 4. Topo II is the target of coumermycin A1. (**A**) DNA gyrase is the target for coumermycin A1. (**B**) DNA gyrase, but not Topo IV, is mainly responsible for norfloxacin-induced HMW DNA fragmentation. Bacterial cells were treated with 50 μM coumermycin A1 for 30 min, then 10 μM norfloxacin was added and incubation continued for another 30 min at 30°C before PFGE was carried out to examine nucleoid integrity. [Cou A1: coumermycin A1; Norf: norfloxacin].

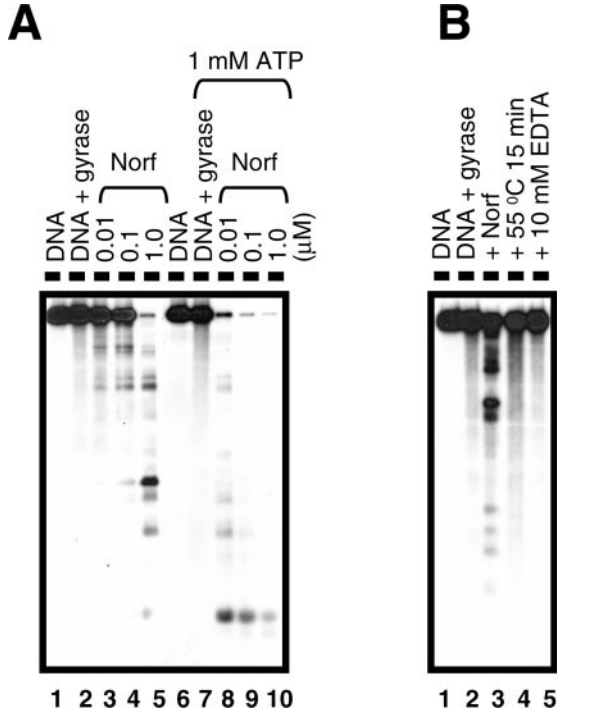

Figure 5. Norfloxacin induces reversible gyrase-mediated DNA double-stranded breaks *in vitro*. (**A**) ATP stimulates gyrase-mediated DNA cleavage in the presence of norfloxacin. Gyrase-mediated DNA cleavage in the presence or absence of 1 mM ATP was examined as described in the Materials and Methods using purified recombinant gyrase. (**B**) Gyrase-mediated DNA cleavage induced by norfloxacin is reversible. Gyrase-mediated DNA cleavage in the presence of 0.1 μM norfloxacin was examined as described in the Materials and Methods. After incubation, duplicate reaction mixtures were subjected for 15 min to different reversal conditions, as indicated at the top of lanes 4 and 5, prior to addition of SDS plus PK.

by either heat reversal (i.e. 55°C for 15 min, Figure 5B, lane 4) or addition of 10 mM EDTA (Figure 5B, lane 5). Taken together, these *in vitro* cleavage experiments provide support for the idea that norfloxacin induces the formation of reversible Topo II cleavable complexes.

Dissection of the distributions of gyrase and Topo IV in nucleoid DNA

Two type II topoisomerases, gyrase and Topo IV, have been identified in *E.coli* and have proposed to have different cellular functions (43,58,59). Gyrase plays a major role in the regulation of supercoiling and Topo IV is the main decatenase for chromosome segregation (43,58,59). In this section, we addressed the relative contributions of gyrase and Topo IV to the norfloxacin-induced excision of HMW DNA fragments, taking advantage of well-characterized norfloxacin-resistant gyrase and/or Topo IV mutant strains [LZ36 (wild-type), LZ35 (*gyrA*), LZ38 (*parC*) and LZ37 (*gyrA*, *parC*)] (43). As shown in Figure 6A, the resistance/sensitivity of these bacterial cells to norfloxacin correlated with their gyrase and/or Topo IV gene status as reported in the literature (43).

Strain LZ35 confers resistance to norfloxacin due to a mutation in gyrase, so the norfloxacin-induced cleavage of HMW DNA fragments in LZ35 nucleoid is mainly through DNA Topo IV proteins. On the other hand, LZ38 cells have a mutation in the DNA Topo IV gene and thus, in these cells, gyrase is the major enzyme causing the cleavage of nucleoid into loop-sized HMW DNA fragments. PFGE analysis revealed that the amount of loop-sized HMW DNA fragments excised from nucleoid in the different strains followed the hierarchy LZ36 > LZ38 $\gg$ LZ35 > LZ37 (Figure 6B), an observation consistent with the notion that DNA gyrase play a more critical role than DNA Topo IV in the formation of loop-sized HMW DNA fragments.

It should be noted that the HMW DNA fragmentation patterns in the different mutants were quite distinct. Figure 6 shows that larger HMW DNA fragments (>200 kb, lane 6) were the main product formed after norfloxacin-induced HMW DNA fragmentation in LZ35 cells (Topo IV-mediated, lane 6), while those formed in LZ38 cells (gyrase-mediated, lane 9) were mainly loop-sized HMW DNA fragments (~50 to 100 kb). This is in agreement with the notion that gyrase play a more critical role in supramolecular DNA loop organization. To summarize, our results showed that, in addition to their biochemical activities, DNA gyrase and topoisomerase IV differ in their locations on nucleoid DNA. Given the fact that gyrase was more active in excision of loop-sized DNA fragments from bacterial nucleoid than Topo IV, it is plausible that gyrase plays a more important role in the maintenance and/or organization of nucleoid supercoiling loop domains (3,6,13,29–31). It should be noted, in agreement with a report by Chen *et al*. (38), that norfloxacin targeted both gyrase and Topo IV *in vivo*.

Roles of type I topoisomerases (TopA and TopB) and SMC proteins in the Topo II-mediated excision of HMW DNA fragments induced by norfloxacin

Topo IIs and DNA topoisomerase I (TopA) are the main enzymes responsible for maintaining the overall supercoiling

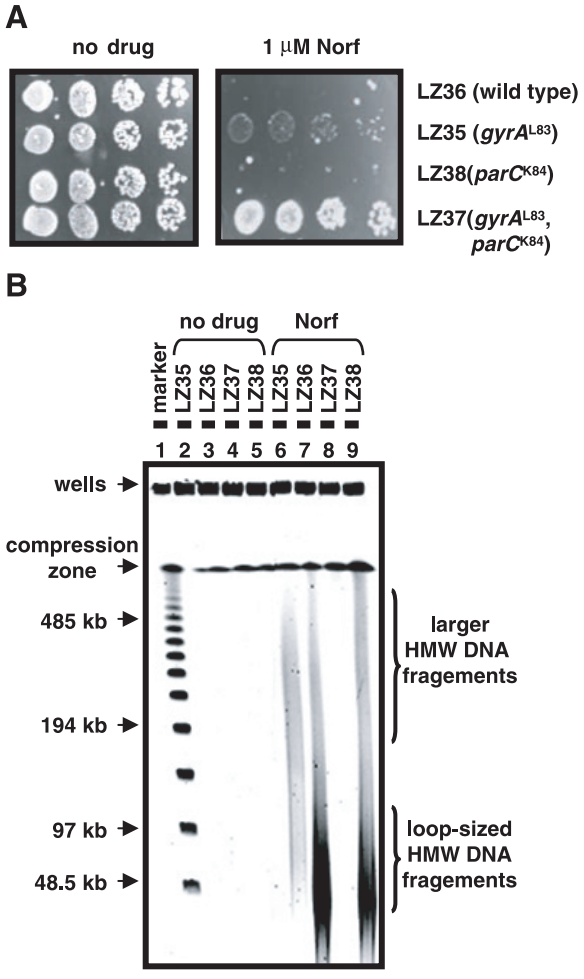

Figure 6. Gyrase plays a more important role than Topo IV in bacterial DNA looped domain organization. (**A**) Differential norfloxacin sensitivities of gyrase and/or Topo IV mutant cells (LZ35 to LZ38). A series of 5-fold dilutions of log-phase cells in LB medium was prepared and 5 μl of each dilution dotted onto LB plates in the presence or absence of 1 μM norfloxacin, then the plates were incubated at 37°C for 16 h and photographed. (**B**) The same strains were grown to log-phase (OD_{595} ~ 0.5), then treated with 10 mM of norfloxacin for 30 min at 37°C. The cells were then encapsulated and their chromosome integrity analyzed by PFGE as described in Figure 1.

state in *E.coli* (40). In addition, both topoisomerase II and SMC proteins have been shown to play an important role in the further condensation of looped structure in the eukaryotic mitotic phase and to be crucial for the faithful segregation of chromosomes in both prokaryotes and eukaryotes (1,60). *E.coli* SMC proteins are encoded by the *mukB*/*E*/*F* genes (1,44). Surprisingly, *topA* mutations were found to suppress the growth and nucleoid organization deficiencies of *muk* mutants (44). We therefore investigated the role of TopA and SMC proteins in the Topo II-mediated excision of HMW DNA fragments induced by norfloxacin.

Consistent with published results (44), only bacterial strain CC4208, carrying a *mukB* mutation, was unable to form colonies at 37°C (Figure 7A). In addition, a *topA* mutation could partially compensate the temperature-sensitive phenotype of the *mukB* mutant (Figure 7A). Furthermore, the

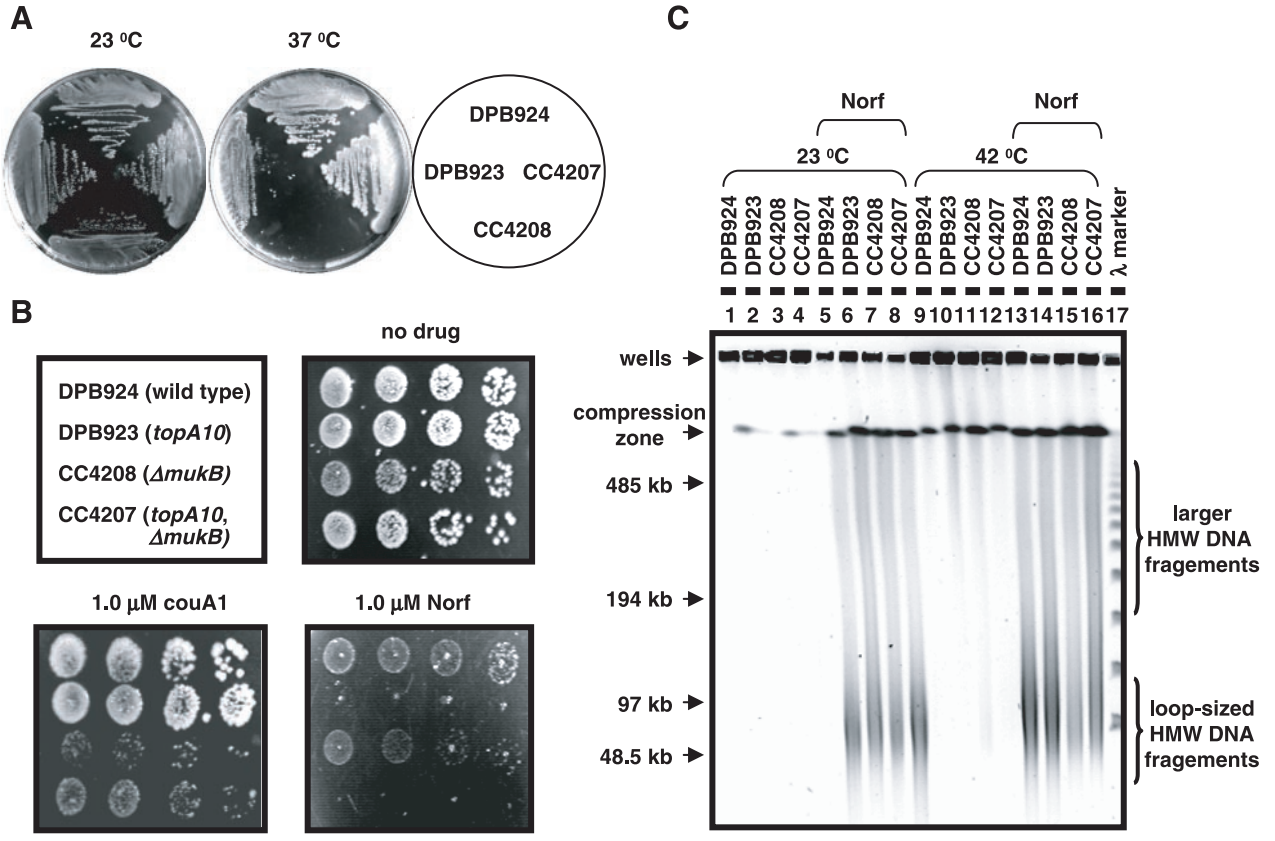

Figure 7. Roles of topoisomerase I (TopA) and/or SMC-like protein (MukB) in nucleoid DNA looped domain organization. (**A**) Temperature sensitivity of the wild-type and *topA* and/or *mukB* mutant strains. Log-phase cells [DPB924 (wild-type), DPB 923 (*topA10*), CC4208 (Δ*mukB*) or CC4207 (*topA10,* Δ*mukB*)] in LB (23°C) were streaked onto LB plates, and then incubated at the indicated temperature for another 36 h. (**B**) Antibiotic sensitivity of the wild-type and *topA* and/or *mukB* mutant strains. A series of 5-fold dilutions of cells grown to log-phase in LB (23°C) was prepared and 5 μl of each dilution dotted onto LB plates containing the indicated antibiotics. Pictures were taken after 36 h incubation at 23°C. (**C**) Strains DPB924 (wild-type), DPB 923 (*topA10*), CC4208 (Δ*mukB*) or CC4207 (*topA10*, Δ*mukB*) were cultured at 23°C to log-phase ($OD_{595} \sim 0.1$), then divided into two groups. The first group was grown at the same temperature until the OD_{595} reaches 0.5. The other group was incubated at a restrictive temperature (42°C) for 2 h to inactive MukB proteins in CC4208 cells. Both sets of cells were then treated for 30 min with 10 mM norfloxacin at the same temperature, encapsulated in agarose plugs, and their nucleoid integrity analyzed as described in Figure 1.

mukB mutant was hypersensitive to coumermycin A1, while the *topA* mutant cells were more sensitive to norfloxacin (Figure 7B).

Using PFGE, we found that norfloxacin-induced less loop-sized fragmentation in the *mukB* strain, which contains a loosed and disorganized nucleoid (44), than in wild-type cells (Figure 7C), especially when the cells were pre-incubated at a restrictive temperature for 2 h before norfloxacin addition (compare lanes 15 and 13). No significant difference in loop excision was seen comparing the wild-type and the *topA* mutant (Figure 7C, lanes 6 and 14). However, *topA* mutation in the *mukB* mutant partially restored norfloxacin-induced loop excision (compare lanes 15 and 16). Our results therefore suggested that SMC proteins, such as MukB, affect the global arrangement of Topo IIs on nucleoid DNA.

E.coli contains four topoisomerases, TopA, gyrase, Topo IV and TopB (topoisomerase III) (40). We therefore examined the effect of topB deficiency on Topo II-mediated cleavage of HMW DNA fragments induced by norfloxacin treatment and found no significant difference in loop excision between the *topB* mutant (CRL3) (61) and the wild-type (W3110) (data not shown). Thus, TopB, like TopA, does not seem to contribute to the distribution of Topo II cleavage sites induced by norfloxacin on nucleoid DNA.

Topo II-mediated excision of nucleoid loop-sized HMW DNA fragments during different growth phases

AFM studies of bacterial nucleoid structure have shown that nucleoid higher-order structure is significantly affected by the growth phase (32). We were therefore interested in determining the patterns of Topo II-excised DNA loops during different growth phases of LZ36 cells. As shown in Figure 8, norfloxacin-induced loop excision was reduced when the cell density reached 4.00 (OD_{595}), an observation consistent with a report that nuclear structure is more highly compacted in stationary phase (32). However, we cannot rule out the possibility that bacterial cells in stationary phase might be more resistant to Topo II-targeting drugs as a result of decreased drug uptake, increased drug efflux, or down-regulation of Topo II.

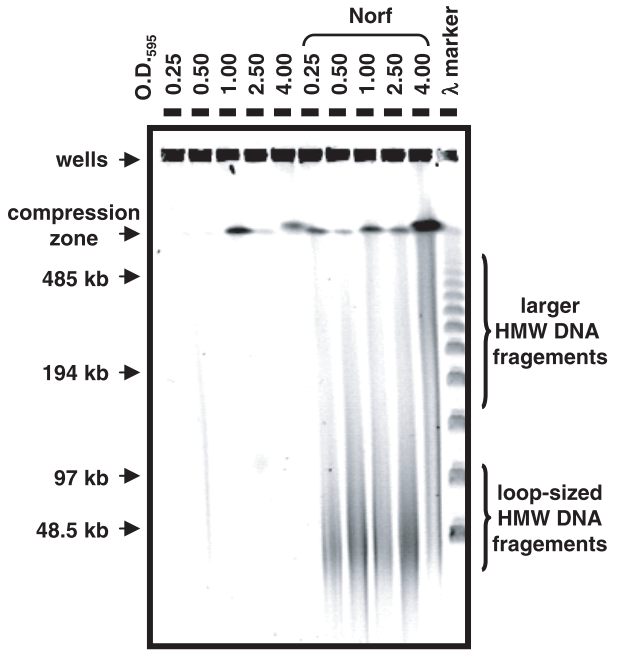

Figure 8. DNA loop organization of nucleoid during different growth phases. LZ36 cells at different growth stages (as indicated by the OD_{595}) were collected, treated for 30 min at 37°C with 10 μM norfloxacin, and subjected to PFGE to analyze the nucleoid looped domains.

DISCUSSION

The length of DNA represents a great topological packaging challenge for any given cell size. In eukaryotic cells, TOP2-mediated excision of DNA loops (~50 to 100 kb) using TOP2-targeting drugs has been extensively used to study the organization of chromosomal long-range structure (11,17,27,28). Here, we found that, in bacteria, norfloxacin-induced Topo II-mediated excision of chromosomal DNA into HMW DNA loop fragments with a non-random, limited loop-size (~50 to 100 kb). We also demonstrated that DNA gyrase, rather than DNA Topo IV, was the main Topo II responsible for the norfloxacin-induced excision of nucleoid DNA into loop-sized HMW DNA fragments. However, consistent with a previous report using sedimentation analysis (38), Topo IV was located at most widely spread sites on nucleoid than gyrase. Our demonstration that bacterial Topo IIs cleaved nucleoid into loop-sized HMW DNA fragments suggests that chromatin structure at the level of the higher-order DNA loop structure is probably conserved in nucleoid DNA in prokaryotes. This notion is supported by early demonstrations of independent supercoiling domains (3,6,13,29–31) and the Topo II-mediated cleavage of nucleoid using oxolinic acid and ciprofloxacin (38,39).

Norfloxacin, oxolinic acid and ciprofloxacin are all quinolone antibiotics. However, the average size of norfloxacin-induced HMW DNA fragments reported in our study (~50 kb), while similar to that of the fragments induced by ciprofloxacin (38), was smaller than for those induced by oxolinic acid (~100 kb) (39). This difference can be explained by the fact that both norfloxacin and ciprofloxacin target gyrase and DNA topoisomerase IV, while oxolinic acid mainly targets gyrase (38,43). It is therefore reasonable to speculate that the smaller DNA fragments generated by norfloxacin or ciprofloxacin are due to additional Topo IV-mediated DNA cleavage of nucleoid DNA. The different distributions of gyrase and Topo IV on nucleoid might well correlate with their different cellular functions. As mentioned above, TOP2-mediated excision of chromosomal DNA loops has been used in mammalian system to probe the structure of the higher-order loop organization of the chromosome. In addition, TOP2 has been suggested to be located at the base of the chromosome loops. We found that gyrase was the main enzyme responsible for the generation of loop-size DNA fragments induced by norfloxacin. It is therefore reasonable to speculate that DNA gyrase, rather than Topo IV, is the Topo II that actively participates in the regulation of loop organization and is located at the base of the nucleoid DNA loops.

Roles of bacterial supercoiling and structuring factors in the distribution of Topo IIs on nucleoid DNA: implications for the organization of bacterial nucleoid

AFM and EM images of nucleoid DNA and other structure-probing assays indicate the existence of non-random DNA organization in the bacterial cell (14,32,62). In addition, earlier reports also suggested the existence of supercoiling-independent domains on a single nucleoid. As in studies of mammalian cells incubated with TOP2-targeting drugs (11,27,28), our study demonstrated that treatment of bacteria with the Topo II-targeting drug, norfloxacin, also induced loop-size DNA fragmentation of the nucleoid. Thus, the radial loop model proposed for higher-order chromatin structure in mammalian chromosomes might also be suitable for the long-range structure of bacterial nucleoid.

Two type II topoisomerases, gyrase and topoisomerase IV, have been identified in *E.coli* (36,40,63). Purified gyrase and Topo IV have different catalytic mechanisms and biochemical activities (43,58,59,64). The major biochemical activity identified for gyrase is the introduction of negative supercoiling, which can efficiently relax the positive supercoiling tension generated from DNA metabolism. Topo IV, on the other hand, does not have great supercoiling activity and is mainly involved in the catenation/decatenation reaction. It is therefore generally believed that gyrase contributes mainly to house-keeping functions involving the regulation of supercoiling during DNA metabolism processes. Topo IV, on the other hand, like mammalian TOP2α, plays a more important role in chromosome segregation. On the basis of the above properties, one would consider that DNA gyrase, rather than Topo IV, would be the enzyme located at the base of DNA loops to regulate the supercoiling tension of topologically independent loops. Our results showing that DNA gyrase was the main enzyme responsible for the norfloxacin-induced excision of nucleoid DNA loops support this notion.

In the present study, we also examined the involvement of MukB, TopA and TopB in the norfloxacin-induced HMW DNA fragmentation of the *E.coli* genome. In agreement with the roles of condensins in eukaryotic higher-order chromatin (1), our results showed that MukB protein was a critical component for the organization of nucleoid DNA loop structure, as indicated by the lack of generation of loop-sized

HMW DNA fragments by norfloxacin in the absence of MukB function, and that TopA and TopB were not. Previous mutational studies have ruled out the involvement of the histone-like proteins, H-NS, HU, FIS and IHF, in the looped domain organization of the nucleoid in *E.coli* (65). However, mutant analysis has also revealed that tight compaction of nucleoid DNA requires Dps (32). In addition, *E.coli* Dps proteins form condensed complexes with DNA (62). It should be interesting to examine the involvement of Dps in the norfloxacin-induced generation of loop-sized DNA fragments.

Cis-elements in the loop organization of bacterial nucleoid: bacterial interspersed mosaic elements (BIMEs) and bacterial TOP2s

As mentioned above, the gyrase and Topo IV cleavage sites examined in our study might well represent the relative distributions of Topo IIs on nucleoid. In addition, the average DNA length of the gyrase-cleaved HMW DNA fragments (~50 kb) in bacteria was similar to the average size (~50 kb) of eukaryotic TOP2-excised chromosomal DNA loops. DNA gyrase might therefore be located on the matrix to which the bases of the DNA loops are attached. What might be the *cis*-DNA elements that interact with gyrase and mediate the attachment of DNA loops to the matrix? In eukaryotic cells, M/SARs on chromosomal DNA can interact with TOP2 and have been proposed to be the *cis*-elements in DNA at the base of chromosomal loops. DNA gyrase is also known to interact with, and cleave, DNA containing BIME sequences (66–68). The BIME family of bacterial highly repetitive DNA elements is found in many species of bacteria (67,69–72). Importantly, the presence of these interspersed DNA sequences located outside the coding regions suggests that BIMEs might have a potential role in defining the compact structure of bacterial nucleoid DNA. BIME sequences contain different repeated motifs and are 40–500 nt in length (67,69–72). Based on specific combinations of different repeated motifs, BIMEs can be further divided into two subfamilies, BIME-1 and BIME-2 (67,69–72). Given our finding that Topo IIs contribute to the organization of nucleoid DNA loop structure, it is tempting to relate the diverse functional properties of BIMEs to those exhibited by nuclear M/SARs, in which eukaryotic TOP2s are located.

Interestingly, the affinities of the BIMEs for DNA gyrase, studied by the gel electrophoretic mobility shift assay (EMSA), have been shown to be different, with BIME-2 having a higher affinity than BIME-1 (66–68). Furthermore, our drug-resistant mutant analysis demonstrated that norfloxacin-induced different patterns of HMW DNA fragmentation mediated by either gyrase or Topo IV. Further clarification of the biochemical interactions between BIMEs and the TOP2s is required.

Recent advances in cytological and genetic methods have suggested that different levels of chromatin structures exist in nucleoid DNA and that several DNA-interacting proteins are components of these chromatin structures (1,8,12,14,32,34,35,73). Chromatin structure and DNA supercoiling are tightly associated with many aspects of cellular function. DNA topoisomerases are actively involved in both processes. Consistent with the above mentioned hypothesis, the patterns of Topo II-mediated cleavage of HMW DNA fragments in the presence or absence of functional SMC proteins were different in the *mukB* mutant. Further studies identifying the relative contributions of TopA, gyrase and Topo IV to nucleoid chromatin organization should help our understanding of nucleoid DNA architecture.

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