JOURNAL OF BACTERIOLOGY, Sept. 1991, p. 5551–5553
0021-9193/91/175551-03$02.00/0

Vol. 173, No. 17

NOTES

Gyrase Inhibitors Increase the Content of Knotted DNA Species of Plasmid pBR322 in *Escherichia coli*

SATOSHI ISHII,† TETSUYA MURAKAMI, AND KAZUO SHISHIDO*

Department of Life Science, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 227, Japan

Received 27 November 1990/Accepted 13 June 1991

Treatment of *Escherichia coli* cells harboring pBR322 with the DNA gyrase inhibitors oxolinic acid and coumermycin A1 led to an increase in the content of knotted pBR322 molecules. This phenomenon was attributed to inhibition of gyrase-catalyzed unknotting of the plasmid DNA knotted by transcription.

A limited amount of information has been obtained about duplex DNA knots produced in vivo. Liu et al. (10) have shown that the majority of the DNAs prepared from tailless capsids of coliphage P2 and P4 are knotted duplex rings. We have found that about 1% of plasmid pBR322 molecules are knotted in *Escherichia coli topA*$^+$ *gyr*$^+$ strains W3110 (18) and DM4100 (16). Strains DM750 (Δ*topA gyrA224*) and DM800 (Δ*topA gyrB225*), carrying a deletion of the DNA topoisomerase I gene with a compensatory mutation of the DNA gyrase gene to reduce enzyme activity (18), and their TopA$^+$ transductants SD275 (*topA*$^+$ *gyrA224*) and SD108 (*topA*$^+$ gyrB225) (4) produced six to eight times as much knotted DNA as the *topA*$^+$ *gyr*$^+$ controls (16). These data suggest that the increased production of knotted pBR322 DNA is closely related to mutations of the gyrase genes.

In this study, we examined the effects of oxolinic acid and coumermycin A1, representatives of two classes of gyrase inhibitors (reviewed in reference 5), on the content of knotted DNA molecules of pBR322. *E. coli* DM800 and DM4100 cells harboring pBR322 were grown to the mid-log phase ($A_{600} \approx 0.7$) at 37°C in LB medium (3) containing L-cysteine (40 μg/ml) and ampicillin (50 μg/ml). The indicated amounts of oxolinic acid (Sigma) or coumermycin A1 (Sigma) were added to the cultures, and they were further incubated for 15 min. To chill the cells quickly before harvesting, we poured the cultures into tubes containing 0.3 volume of frozen LB medium; under these conditions, culture temperatures reached about 6°C within about 3 min. pBR322 DNA was isolated from pelleted cells by the alkaline lysis method described by Maniatis et al. (11), except that lysozyme treatment was done on ice for 10 min instead of at room temperature for 5 min. About 30 min usually elapsed between the time the cells were removed from 37°C and the time lysis was complete. pBR322 DNA from strains DM800 and DM4100 was nicked by pancreatic DNase I and electrophoresed in a 1% agarose slab gel by the published procedure (16), resulting in separation of knotted nicked DNA species with various numbers of nodes from the unknotted nicked circular form. The DNA bands separated were transferred to a nylon membrane (Hybond-N; Amersham) by the method of Reed and Mann (14) and detected by Southern blot hybridization (17) with a ^{32}P-labeled pBR322 probe.

Analysis of the knotted DNA molecules of pBR322 showed that predominantly knots with an odd number of nodes were produced (Fig. 1a1 and a2), confirming previous results (15, 16). In DM800 (Fig. 1a1), both oxolinic acid (lanes 1 and 2) and coumermycin A1 (lanes 3 and 4) were found to increase the amount of pBR322 knotting (compare with lane C). Oxolinic acid displayed an especially dramatic effect at the higher concentration (5 μg/ml) (lane 2). The hybridization bands were cut out from the membranes, and their radioactivities were counted. pBR322 DNA isolated from DM800 cultured in the presence of 0.5 and 5 μg of oxolinic acid per ml or 1 and 15 μg of coumermycin A1 per ml was estimated to contain about 9 and 17% or 9 and 10% of the knotted forms, respectively. In the same way, the amount of the knotted form of pBR322 DNA cultured in the absence of gyrase inhibitor was estimated to be 5.5%. In DM4100 (Fig. 1a2), a higher concentration of gyrase inhibitors also increased the amount of knotted pBR322 DNA, although the amount of pBR322 knotting was low compared with that in DM800. In both DM800 and DM4100, increasing the concentrations of oxolinic acid and coumermycin A1 to 50 and 100 μg/ml, respectively, led to only a little additional accumulation of knotted pBR322 molecules compared with the results with 5 μg of oxolinic acid per ml and 15 μg of coumermycin A1 per ml (data not shown).

To interpret the results stated above, we assessed the state of negative supercoiling of the pBR322 DNA used for measuring the amount of knotted DNA by chloroquine-agarose gel electrophoresis (Fig. 1b1 and b2). In these panels, the lowest pBR322 band (marked with an arrow) is the most supercoiled, the intermediate bands are less supercoiled, and the top band (marked with an arrow) is relaxed (nicked) circular DNA. The distribution of topoisomers shifts upward with less supercoiling. pBR322 DNA isolated from DM800 was extremely heterogeneous and highly negatively supercoiled (Fig. 1b1, lane C), and that from DM4100 showed much lower negative superhelicity (Fig. 1b2, lane C), as reported previously (12, 13, 15). Like the data obtained by other investigators (5), a higher concentration of gyrase inhibitors reduced the negative superhelicity of pBR322 DNA in DM800 (Fig. 1b1) and DM4100 (Fig. 1b2) through interference with the action of gyrase; the reduction of superhelicity by 5 μg of oxolinic acid per ml in DM800 was

* Corresponding author.
† Present address: Life Science Research Laboratory, Japan Tobacco Inc., 6-2 Umegaoka, Midori-ku, Yokohama 227, Japan.

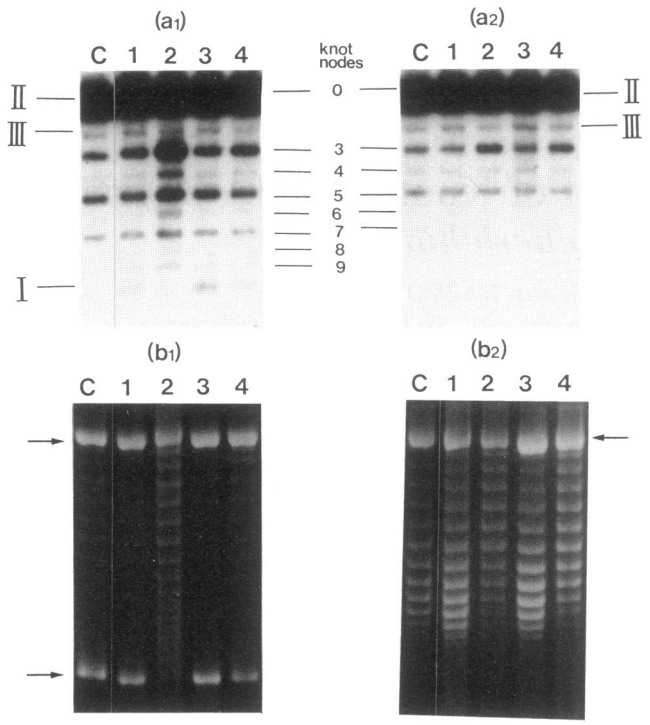

FIG. 1. Agarose gel electrophoretic analyses of the effects of DNA gyrase inhibitors on the content of knotted DNA species of pBR322 (a1 and a2) and on the degree of pBR322 supercoiling (b1 and b2) in *E. coli*. (a1 and a2) Samples (0.2 μg each) of pBR322 isolated from DM800 (a1) and DM4100 (a2) were cultured in the absence (lane C) or presence of 0.5 μg (lane 1) and 5 μg (lane 2) of oxolinic acid per ml or 1 μg (lane 3) and 15 μg (lane 4) of coumermycin A1 per ml. The DNA probe was labeled with [α-^{32}P]dCTP (Amersham) by the method of Feinberg and Vogelstein (6). Hybridization was performed at 42°C for 24 h in a solution containing 25 mM sodium phosphate buffer (pH 6.5), 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 0.5% sodium dodecyl sulfate (SDS), and 100 μg of heat-denatured herring sperm DNA per ml. The membranes were washed twice in 2× SSC containing 0.05% SDS at 42°C and autoradiographed. The numbers of knot nodes assigned in a previously published report (16) are given to each the DNA bands. Unknotted nicked circular (II) and unit-length linear (III) forms of pBR322 and a trace amount of supercoiled pBR322 DNA (I) that escaped the nicking treatment are indicated. (b1 and b2) Samples (1.5 μg each) of pBR322 identical to those of panels a1 and a2 were electrophoresed before nicking on a 1% agarose slab gel containing chloroquine (12 μg/ml) under the published conditions (12).

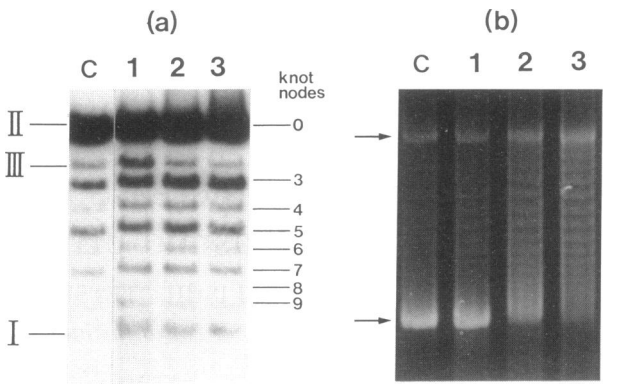

FIG. 2. Effect of oxolinic acid on the content of knotted pBR322 DNA (a) and the degree of pBR322 supercoiling (b) in DM800 as a function of the time of addition of the drug. Oxolinic acid (5 μg/ml) was added when the cell culture was chilling (lane 1), or the cell culture was treated with the drug for 7.5 min (lane 2) or 15 min (lane 3) and then rapidly chilled. A cell culture untreated with the drug (lane C) was prepared in parallel. Samples of pBR322 were analyzed as described in the legend to Fig. 1.

especially significant (Fig. 1b1, lane 2). Hence, it appears that inhibition of DNA gyrase ability is closely related to the increase in the amount of knotted pBR322 DNA. The inhibitors may prevent the gyrase-catalyzed unknotting of knotted plasmid DNA. One might wonder why the effect of oxolinic acid is much higher than that of coumermycin A1. A possible answer for this question is as follows. A variant of DNA gyrase with almost one-half of the B subunit missing, DNA topoisomerase II′ (1, 2, 7), also takes part in the unknotting reaction. Unlike gyrase, it can unknot the knotted DNA in an ATP-independent manner. So coumermycin A1, which binds to the B subunit of gyrase and blocks its ATPase activity (5), does not inhibit the unknotting reaction catalyzed by topoisomerase II′. Oxolinic acid, which interacts with the A subunit of gyrase and arrests the breakage-rejoining cycle with the enzyme stuck on the DNA (5), prevents the topoisomerase II′-catalyzed unknotting reaction.

The effect of oxolinic acid (5 μg/ml) on pBR322 knotting in DM800 was tested by varying how long the cell culture was treated at 37°C; the inhibitor was added to the culture when it was chilling (0-min treatment), or the culture was treated with the inhibitor for 7.5 min instead of 15 min and then rapidly chilled. As shown in Fig. 2a, an increase in the amount of knotted pBR322 DNA was observed with the 0-min treatment (lane 1) as well as with the 7.5-min treatment (lane 2). The extents of increase with the 0-min and 7.5-min treatment were 2.8 and 3.1 times that of the untreated control (lane C), respectively, being almost the same as that (3.1 times) of the 15-min treatment (lane 3). pBR322 DNA from the 0-min-treatment cells showed a high level of negative supercoiling distribution similar to that from the untreated control cells (lanes 1 and C of Fig. 2b). pBR322 DNA from the 7.5-min- and 15-min-treatment cells showed a decreased level of negative supercoiling (Fig. 2b, lanes 2 and 3). These data, together with the results of Fig. 1, may be explained as follows.

In cultured *E. coli* cells, both knotting and unknotting reactions occur on pBR322 DNA, and they are controlled to maintain the amount of knotted DNA molecules at a constant level. This level, however, is different between DM800 and DM4100. Our previous report (15) showed that transcription of the tetracycline resistance gene sequence of pBR322 is a major determinant of the level of plasmid knotting. So strains DM800 and DM4100 were treated with a sufficient amount of rifampin, an inhibitor of initiation of RNA synthesis, to block transcription (prevent the knotting process) for 15 min before the cells were harvested. No production of knotted pBR322 DNA was observed in either strain (Fig. 3), showing that the unknotting reaction occurred exclusively. Even in the presence of gyrase inhibitor, the amount of knotted pBR322 DNA showed little change in cultured cells; otherwise, pBR322 DNA from the 0-min-treatment cells would display an intermediate level of plasmid knotting. A variation in the amount of knotted DNA in

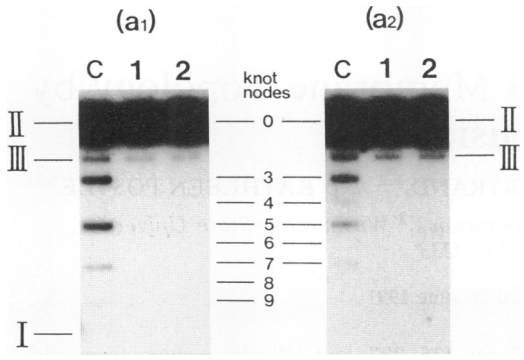

FIG. 3. Elimination of knotted pBR322 DNA production in *E. coli* by rifampin treatment. Samples (0.2 μg) of pBR322 isolated from DM800 (a1) and DM4100 (a2) were cultured in the absence (lane C) or presence (25 μg/ml, lane 1; 50 μg/ml, lane 2) of rifampin (Sigma) for 15 min before the cells were harvested. The cells were analyzed as described in the legend to Fig. 1.

each pBR322 sample may originate from a difference in the degree of blocking of the unknotting reaction, which proceeds in about 30 min between the time the cells are removed from 37°C and the time lysis is complete. Oxolinic acid blocked the unknotting reaction even if it was added to the chilled culture. In the above 30 min, however, negative superhelical turns of pBR322 were nearly not removed.

Concerning the knotting reaction of pBR322 DNA, we do not have a clear answer now. However, DNA gyrase is known to occasionally knot DNA in vitro (9). Kato et al. (8) have recently isolated a new type II topoisomerase, named topoisomerase IV, essential for chromosome segregation in *E. coli*. This enzyme is also a potential producer of knotted DNA.

This study revealed that in growing *E. coli* Δ*topA* cells, some knotted pBR322 molecules are generated, exposing a new aspect in the intracellular topological DNA structure of plasmid.

We thank R. Sternglanz and J. Wang for kindly providing DM800 and DM4100, respectively.

This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. **Adachi, T., M. Mizuuchi, E. A. Robinson, E. Appella, M. H. O'Dea, M. Gellert, and K. Mizuuchi.** 1987. DNA sequence of the *E. coli gyrB* gene: application of a new sequencing strategy. Nucleic Acids Res. **15**:771–783.
2. **Brown, P. O., C. L. Peebles, and N. R. Cozzarelli.** 1979. A topoisomerase from *Escherichia coli* related to DNA gyrase. Proc. Natl. Acad. Sci. USA **76**:6110–6114.
3. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. A manual for genetic engineering. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
4. **DiNardo, S., K. A. Voelkel, and R. Sternglanz.** 1982. *Escherichia coli* DNA topoisomerase I mutants have compensatory mutations in DNA gyrase genes. Cell **31**:43–51.
5. **Drlica, K., and R. J. Franco.** 1988. Inhibitors of DNA topoisomerases. Biochemistry **27**:2253–2259.
6. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
7. **Gellert, M., L. M. Fisher, and M. H. O'Dea.** 1979. DNA gyrase: purification and catalytic properties of a fragment of gyrase B protein. Proc. Natl. Acad. Sci. USA **83**:7152–7156.
8. **Kato, J., Y. Nishimura, R. Imamura, H. Niki, S. Hiraga, and H. Suzuki.** 1990. New topoisomerase essential for chromosome segregation in *E. coli*. Cell **63**:393–404.
9. **Kreuzer, K. N., and N. R. Cozzarelli.** 1980. Formation and resolution of DNA catenanes by DNA gyrase. Cell **20**:245–254.
10. **Liu, L. F., L. Perkocha, R. Calender, and J. C. Wang.** 1981. Knotted DNA from bacteriophage capsids. Proc. Natl. Acad. Aci. USA **78**:5498–5502.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Pruss, G. J.** 1985. DNA topoisomerase I mutants: increased heterogeneity in linking number and other replicon-dependent changes in DNA supercoiling. J. Mol. Biol. **185**:51–63.
13. **Pruss, G. J., and K. Drlica.** 1986. Topoisomerase I mutants: the gene on pBR322 that encodes resistance to tetracycline affects plasmid DNA supercoiling. Proc. Natl. Acad. Sci. USA **83**: 8952–8956.
14. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13**:7207–7221.
15. **Shishido, K., S. Ishii, and N. Komiyama.** 1989. The presence of the region on pBR322 that encodes resistance to tetracycline is responsible for high levels of plasmid DNA knotting in *Escherichia coli* DNA topoisomerase I deletion mutant. Nucleic Acids Res. **17**:9749–9759.
16. **Shishido, K., N. Komiyama, and S. Ikawa.** 1987. Increased production of a knotted form of plasmid pBR322 DNA in *Escherichia coli* DNA topoisomerase mutants. J. Mol. Biol. **195**:215–218.
17. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
18. **Sternglanz, R., S. DiNardo, K. A. Voelkel, Y. Nishimura, Y. Hirota, K. Becherer, L. Zumstein, and J. C. Wang.** 1981. Mutations in the gene coding for *Escherichia coli* DNA topoisomerase I affect transcription and transposition. Proc. Natl. Acad. Sci. USA **78**:2747–2751.