An infrequent generation of catenated network of pBR322 in *Escherichia coli*

Naoki Komiyama and Kazuo Shishido*

Department of Life Science, Faculty of Science, Tokyo Institute of Technology, Nagatsuta, Yokohama 227, Japan

Received 15 May 1986

It was demonstrated that *Escherichia coli* infrequently generates the catenated network of pBR322. This complex pBR322 form was detected when DNA molecules could hardly enter the agarose gel during electrophoresis and was found to comprise monomers and dimers of the plasmid.

Catenated network (E. coli) *Plasmid pBR322* *Monomer* *Dimer*

1. INTRODUCTION

When bacterial plasmid DNA prepared by removing the cell DNA, RNA and protein and bacterial debris is electrophoresed in agarose gel before purification of the plasmid DNA by equilibrium centrifugation in CsCl ethidium bromide (EtBr) density gradient, the DNA fraction which hardly enters the gel is usually detectable. So far, this DNA species has not been analyzed in detail, as far as we know. We have isolated this DNA species from the preparation of plasmid pBR322 produced in *Escherichia coli* and analyzed it by enzymatic digestion followed by electron microscopic examination. The results have revealed that a part of the DNA molecules hardly entering the gel is a catenated network of pBR322. In this paper, we wish to report the details of the experiments.

2. MATERIALS AND METHODS

2.1. *Transformation and cultivation of* E. coli *and preparation of pBR322 DNA*

E. coli W3110 (wild type) was transformed with purified monomeric pBR322 DNA according to Davies et al. [1]. LB medium containing 50 μg ampicillin/ml was used to grow the transformants carrying the plasmid. Cells from an overnight culture were diluted 100-fold into fresh growth medium (200 ml or 1 l), grown at 37°C with aeration to late exponential phase ($A_{600} \sim 0.7$), quickly chilled and centrifuged. pBR322 DNA was prepared basically by alkaline extraction procedure as described in [2]. To the alkali-lysed cells potassium acetate (pH 4.8) was added to neutralize it. After standing on ice for 10 min, the mixture was centrifuged to remove the cell DNA and bacterial debris. 0.6 vol. isopropanol was added to the supernatant and kept at room temperature for 15 min, and the DNA was recovered by centrifugation. The nucleic acid pellet was washed with 70% ethanol, dried and dissolved in TE buffer (10 mM Tris-HCl (pH 7.5)/1.0 mM EDTA) to give a final conc. ~ 100 μg/ml. The DNA solution was incubated with RNase A (Sigma, 50 μg/ml) at 37°C for 1 h followed by digestion with proteinase K (Boehringer Mannheim, 100 μg/ml) at 37°C for 1 h. The reaction mixture was extracted three times with phenol, treated with diethyl ether, and the DNA precipitated with ethanol. The DNA pellet was dissolved in TE buffer and again treated with proteinase K under the above conditions. The reaction mixture was deproteinized with phenol, and the ethanol-precipitated DNA was dissolved in TE buffer followed by dialysis against TE buffer. The resulting pBR322 DNA preparation was subjected to agarose gel electrophoresis.

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

2.2. *Enzymes and reactions*

The indicated amount of substrate DNA was incubated with 5 units of S1 at 37°C for 30 min in the reaction buffer (20 μl) containing 45 mM sodium acetate buffer (pH 5.0), 1 mM $ZnSO_4$, 1 mM $MnCl_2$ and 70 mM NaCl. The reaction was stopped by addition of Tris-HCl (pH 8.5) and EDTA to give a final conc. 100 and 25 mM, respectively. 1 unit of S1 activity is defined as the amount of the enzyme that converts 50% of 0.5 μg single-stranded pBR322 DNA to acid-soluble form under the above conditions. Restriction endonucleases were purchased from Takara Shuzo, Kyoto, Japan and used according to the supplier's instruction.

2.3. *Agarose gel electrophoresis*

Horizontal 1% agarose slab gels were prepared and electrophoresed in Tris-acetate buffer, pH 8.2 (40 mM Tris-base/20 mM sodium acetate/1 mM Na_2EDTA) at 6 V/cm for 3–4 h. The DNA bands were stained with EtBr, visualized using short-wavelength UV light and then photographed as in [3].

2.4. *Electron microscopy*

DNA sample was nicked by pancreatic DNase I (Worthington) as described in [4]. After deproteinization with phenol, the purified DNA sample was subjected to electron microscopic analysis. After spreading onto carbon-coated grids, the DNA was stained with uranyl acetate, shadowed with platinum-palladium, and visualized with a JEM 100CX electron microscope.

3. RESULTS AND DISCUSSION

Fig.1 shows the agarose gel electrophoretic patterns of pBR322 DNA prepared from the separately cultured cells in each of two *E. coli* W3110 transformants (lanes 2–5, and lanes 6 and 7) as described in section 2. In all pBR322 preparations, a small portion of the DNA was shown to hardly enter the gel. The amount of this DNA fraction was estimated by densitometric measurement to be 1–2% of the total plasmid DNA (the DNA includes a slight amount of the cell DNA), while pBR322 finally purified by equilibrium centrifugation in a CsCl-EtBr density gradient was found not to contain the DNA which hardly enters the gel (lane 1).

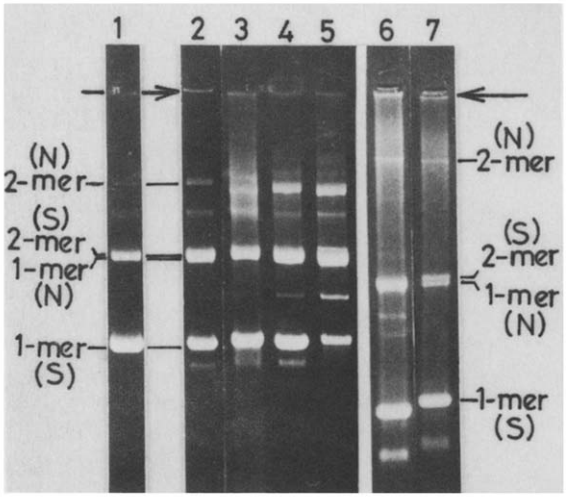

Fig.1. Agarose gel electrophoretic patterns of various preparations of pBR322 from *E. coli* W3110. Approx. 5 μg each of pBR322 preparations was electrophoresed. Lanes: 2–5, four independent preparations from one transformant; 6 and 7, two independent preparations from another transformant. pBR322 (isolated from W3110) was finally purified by equilibrium centrifugation in a CsCl-EtBr density gradient as described in [2] and electrophoresed as a reference (lane 1). Arrows, the position of the DNA species that hardly enters the gel. 1-mer and 2-mer, monomer and dimer, respectively. S and N, supercoiled and nicked forms, respectively.

The above novel DNA species was recovered from the gel and heated at 65°C for 10 min in TE buffer containing 10 mM NaCl followed by rapid cooling. The resulting DNA was electrophoresed. Approx. 30% of the DNA still showed the property of hardly entering the gel (lane 1 in fig.2a). The other DNA was converted to a monomer and dimer of pBR322 and to a slight amount of oligomer of the plasmid, suggesting the DNA to be a complex pBR322 molecule which may be artificially connected by intermolecular base pairing through alkali-denaturation and neutralization of DNA in the preparation of pBR322 (see section 2). The DNA hardly entering the gel was recovered, heated and cooled, and then re-electrophoresed. All the DNA was found to retain the novel property (lane 2 in fig.2a). This DNA was digested with restriction endonuclease *Bam*HI which cleaves pBR322 at a single unique site [5]. As shown in fig.2b, the DNA was converted to a linear DNA of size identical to a pBR322 *Bam*HI-digest. The

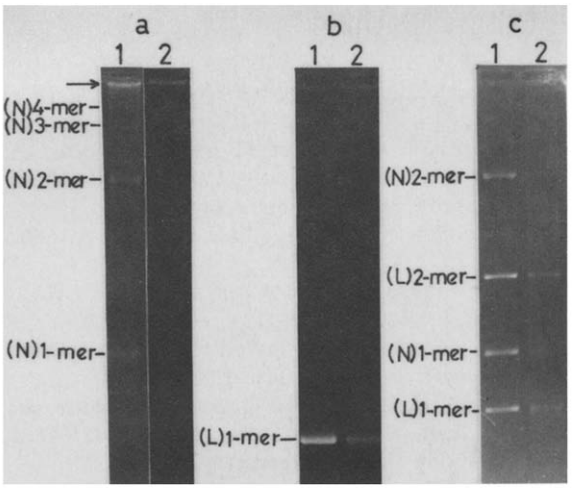

Fig.2. Agarose gel electrophoretic patterns of the DNA that hardly enters agarose gel (a) after treatment with *Bam*HI (b) and S1 nuclease (c). The DNA hardly entering the gel was recovered as follows. About 1 mg of pBR322 was electrophoresed in low melting point agarose (BRL) as in section 2. The gel containing the DNA band was cut out and melted by incubation at 62°C for 15 min. 3 vols TE buffer were added and further incubated at 62°C for 20 min. The incubation mixture was extracted three times with an equal volume of phenol, treated with diethyl ether and precipitated with ethanol. The DNA pellet dialyzed against TE buffer. (a) Details are in the text. Lane 1, 1 μg; lane 2, 0.5 μg. In b and c: lane 1, the CsCl-purified pBR322 DNA (2 μg); lane 2, the DNA species hardly entering the gel (0.5 μg). 3-mer and 4-mer, trimer and tetramer, respectively. L, unit-length linear form.

analyses using the other restriction endonucleases such as *Taq*I and *Hin*fI which cleave pBR322 at many sites confirmed that the DNA is a certain form of pBR322 itself.

Covalently closed, circular plasmid DNA is known to possess the non basepaired sites introduced by its negative superhelicity. S1, a single-strand specific nuclease, can cleave in either one of two strands once at the non basepaired sites and then the nicked, circular DNA is cleaved on the opposite strand at the nick to yield the unit-length linear molecule [6]. S1 can also cleave the opposite strand at a naturally occurring nick. Thus the novel DNA form of pBR322 was analyzed using S1 nuclease. The patterns given in fig.2c exhibited the conversion of the DNA to monomeric and dimeric linear (and nicked circular) pBR322 forms, indicating that the DNA hardly entering the gel comprises monomers and dimers of pBR322.

Previously, other investigators have noticed the presence of DNA molecules that hardly entered the agarose gel in the reaction mixture of plasmid DNA and *E. coli* DNA gyrase or *Drosophila* type II DNA topoisomerase, and those DNA molecules were identified to be catenated DNA network [4,7]. So, our analogous DNA species of pBR322 isolated from *E. coli* cells were subjected to electron microscopic analysis; the DNA was nicked before spreading to eliminate supertwists and thereby simplify interpretation. As a reference, a nicked monomer circle of pBR322 was also spread. The only molecules seen were a net-structure of DNA (see fig.3). This net-structure, as mentioned above, was resolved by S1 nuclease into monomer and dimer linear pBR322 forms, indicating that the DNA is the catenated network consisting of monomers and dimers of pBR322. The total length of the catenated network seems to be seven or more times the monomeric unit of pBR322.

In this paper, it has been demonstrated that a catenated network of pBR322 is produced in *E. coli* cells. However the rate of its production is very low, being only about 0.5% of the total plasmid DNA. The copy number of pBR322 in *E. coli* W3110 is estimated to be around 30 under the conditions used here. Therefore the frequency of the production of the catenated network in *E. coli* W3110 is calculated to be approx. 1 per 40 cells

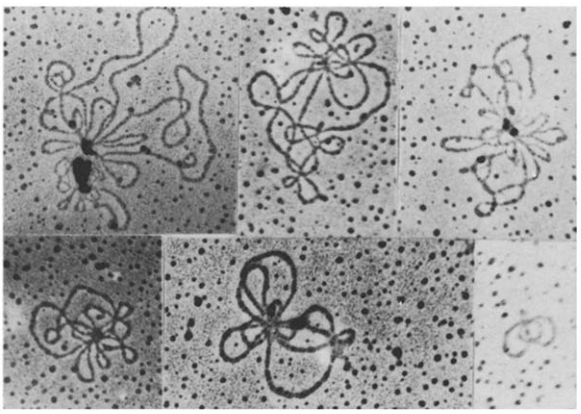

Fig.3. Electron micrographs of the DNA molecules hardly entering agarose gel. Nicked monomer pBR322 is seen in the bottom, futhest right panel.

(based on the assumption that the total length of the catenated network is 7-times a monomeric unit). We have examined other *E. coli* strains including DNA topoisomerase mutants (such as DM750 and SD275 that appeared in [8]) for the rate of production of the catenated network of pBR322. The data showed that its rate is similar in all the strains (not shown).

The above results indicate that plasmid generation in *E. coli* is well controlled to prohibit the formation of the plasmid with an abnormal structure.

ACKNOWLEDGEMENT

We are grateful to Dr N. Noguchi of the Tokyo College of Pharmacy for technical assistance in the electron microscopic examination of DNA.

REFERENCES

[1] Davies, R.W., Botstein, D. and Roth, J.R. (1980) Advanced Bacterial Genetics, Cold Spring Harbor Laboratory, NY.
[2] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, NY.
[3] Shishido, K. (1979) J. Biochem. (Tokyo) 86, 711–717.
[4] Hsieh, T. (1983) J. Biol. Chem. 258, 8413–8420.
[5] Bolivar, F., Rodrigues, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S. (1977) Gene 2, 95–113.
[6] Shishido, K. and Ando, T. (1982) in: Nucleases (Linn, S.M. and Roberts, R.J. eds) pp. 155–185, Cold Spring Harbor Laboratory, NY.
[7] Kreuzer, K.N. and Cozzarelli, N.R. (1980) Cell 20, 245–254.
[8] DiNardo, S., Voelkel, K.A. and Sternglanz, R. (1982) Cell 31, 43–51.