Positively supercoiled plasmid DNA is produced by treatment of *Escherichia coli* with DNA gyrase inhibitors

Daniel Lockshon and David R. Morris

Department of Biochemistry, University of Washington, Seattle, WA 98195, USA

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ABSTRACT

A procedure has been developed whereby the relative amounts of the topoisomers of *E. coli* plasmid can be determined for cells grown under a variety of conditions. Several applications of the procedure are presented. Addition of either novobiocin or oxolinic acid, two inhibitors of DNA gyrase, gives rise to positively supercoiled plasmid. A likely model for the introduction of positive supercoils, involving DNA gyrase itself, is discussed. Oxolinic acid is also shown to induce linearization of plasmid *in vivo*. Starvation of cells for ATP is shown to cause relaxation of plasmid. The shift of a *gyrB* temperature-sensitive strain to the restrictive temperature is also shown to cause plasmid relaxation. Finally, it is noted that polyamine starvation of *E. coli* has no detectable effect on the distribution of topoisomers.

INTRODUCTION

The superhelical density of prokaryotic DNA has been shown to be relevant to its replication [1,2], transcription [3,4], recombination [5] and transposition [6]. Studies with intact cells have been limited to the analysis of chromosomal DNA superhelicity. There are several advantages in examining the supercoiling of plasmid DNA rather than that of chromosomal DNA. Plasmid supercoiling is far easier to analyze since plasmid can be manipulated easily and its topoisomers can be resolved by agarose gel electrophoresis [7]. A correlation between supercoiling and various cellular processes can also be more conclusively demonstrated with plasmid than with chromosome, since plasmid is believed to contain only a single topological domain *in vivo* whereas chromosome contains 43 ± 10 [8]. Thus, plasmid superhelicity is invariant throughout a given molecule, whereas chromosome supercoiling is an average value which could vary from domain to domain. Therefore, if one were interested in examining whether DNA supercoiling correlates with the rate of replication fork movement, for example, measurement of chromosome supercoiling would not necessarily indicate the supercoiling of the domain(s) containing the replication forks.

The present study details a method which can analyze the population of plasmid topoisomers (the "topoprofile") from *Escherichia coli* in various physiological states. In addition to presenting the method and experiments which demonstrate its efficacy, we

also present several applications. Most notably we have found that novobiocin (NOV*) and oxolinic acid (OXO), representatives of two classes of inhibitors of DNA gyrase [9], not only permit relaxation of the negative supercoils normally maintained in plasmid, but induce the cell to generate positive supercoils as well. We show that OXO also induces plasmid linearization, as one would expect from *in vitro* studies [10,11,12]. In addition, we examine the effects on plasmid topoprofile of ATP starvation, of shifting a *gyrB*^{ts} strain to the restrictive temperature, and of starving an *E. coli* auxotroph for polyamines.

MATERIALS AND METHODS

Materials

NOV (Sigma reagent, slightly yellow crystals, not further purified) was used as a freshly made 10 mg/ml solution. Coumermycin, from Godfrey Science, Syracuse NY, was used as a DMSO solution of 10 mg/ml. OXO was a gift of Dr. R. D. Westland of Parke-Davis. It was used as a 1 mg/ml solution adjusted to pH 9.0 with NaOH. 2,4-dinitrophenol (DNP), purchased from Sigma and recrystallized prior to use, was used as a 1 M solution in DMSO. DNase I was from Worthington. *E. coli* Topoisomerase I (ω protein) was kindly provided by Dr. J. C. Wang.

Nuclear extract from either Sp2/0 or Chinese Hamster Ovary cell lines was made as follows: Cells (3×10^7) were lysed in 10 mM Tris-HCl, 1 mM EDTA, 4 mM MgCl₂, 2 mM DTT (pH 7.5) using a Dounce homogenizer. After isolation of the nuclei by centrifugation, they were sonicated in 5 ml of cold 20 mM Tris-HCl, 1 mM EDTA, 0.5 mM DTT (pH 7.5) until most were judged by microscopic inspection to have burst. The supernatant solution from a subsequent centrifugation for 10 min at 27,000 x g was stored at -80° for 2 years without significant loss of relaxation activity.

Bacteriological procedures

Strain DK6recA⁻(pBR322) was used unless otherwise indicated. LE316 [13] was kindly provided by Dr. Elisha Orr. EWH319 [14] was provided by Dr. Herbert Tabor. pBR322 [15] was the only plasmid used in this work. Transformation with plasmid was done according to standard procedures. When DK6 [16] contained recA⁺, we observed large amounts of plasmid multimerization and this was completely prevented by the introduction of recA56 from JC10240 (provided by Dr. A. J. Clark) using P1 phage transduction [17]. Cells were grown at 37° (unless otherwise noted) with vigorous shaking in MOPS-buffered medium [18] supplemented with 1.3 mM potassium phosphate, 0.2% glucose, 0.001% spermidine, and a synthetic amino acid mixture [19] to a density of $A_{450} = 0.15 \pm 0.05$.

Cell lysis procedure

One ml of bacterial culture was removed from its flask and added to a vortexing

Falcon 2001 tube containing 1 ml of 2.5% SDS, 2 mM EDTA which had been kept at 42°. Within 1 min, 50 µl of pronase B solution (Calbiochem; a fresh solution of 10 mg/ml was autodigested 2 hr at 37° prior to use) was added with vortexing. The samples were left overnight at room temperature. To rid the sample of SDS, which interferes with its manipulation, 1 ml of lysate was added to 0.1 ml of 1 M KCl and the white precipitate was removed by centrifugation. The supernatant solution was removed and 80 µl of it was run on an agarose gel which was then blotted and probed as described below.

Gel electrophoresis, blotting, and hybridization

All gels were 2.1% agarose (SeaKem ME) in 50 mM Tris-phosphate and 1 mM EDTA (pH 7.5). They were cast with dimensions 6 x 145 x 188 mm and run horizontally. The apparatus, designed by the authors, had reservoirs of 1400 ml capacity which made electrical contact with the gel by two cheesecloth wicks. Gels were run 20 hr at 120 V with circulating running buffer. When chloroquine phosphate (from Sigma) was used, it was added to the buffer before the gel was cast. Following the run, the gel was X-irradiated (100 krad) to nick the circular DNA strands. The gel was then trimmed and soaked for 1 hr, with shaking, first in 500 ml 0.5 N NaOH containing 1.5 M NaCl and then in 500 ml 1 M Tris-HCl and 2 M NaCl (pH 5.5). Blotting of the gel onto slightly oversized nitrocellulose (S & S, BA85) was done using 20x SSC and Southern's method [20]. The blot was washed and baked as described [20]. One or two blots were placed in a sealed plastic bag, incubated overnight in 40 ml 10X Denhardt's solution [20], 6X SSC, 20 mM Tris-HCl (pH 7.5) and then for 4 to 6 hr in 10 ml 5X Denhardt's solution, 3X SSC, 20 mM Tris-HCl, 2 mM EDTA, 0.5% SDS, 1 µg calf thymus DNA per ml at 68°. Nick-translated ³²P-labelled plasmid was then added and hybridization was carried out overnight at 68° with intermittent shaking. In order to insure probe excess and a linear response, at least 0.3 µg of probe (3 x 10⁷ cpm) was added. The blots were then washed as described [20], dried, and exposed to Agfa Osray RPI film for 1 to 7 days. All the figures (except Fig. 2) are reproductions of such autoradiograms and are oriented so that DNA migration was from top to bottom.

Preparation of topological markers

A plasmid population, which was defined as having the center of its distribution at $\tau^\circ = 0$, was compared with various populations of progressively more negatively supercoiled populations by band-counting in gels containing various levels of chloroquine. The $\tau^\circ = 0$ standard was produced by complete relaxation in the standard buffer suggested by Anderson and Bauer [21] at 37° using purified rat liver type 1 topoisomerase (kindly provided by Dr. J. J. Champoux). The other relaxation reactions, containing 7 µg pBR322 per ml, 170 µl CHO cell nuclear extract per ml, 0.17 M KCl, 40 mM Tris-HCl, 1 mM EDTA, and 0, 0.5, 1, 1.5, 2 or 3 µM ethidium

Figure 1: Prevention of OXO-induced plasmid linearization by addition of lysis solution. Lanes are identified by the amount of time (in sec) of exposure of cells to OXO before addition of lysis solution, or by "ctrl" to indicate omission of drug treatment. For the positively numbered lanes, OXO (30 $\mu\text{g/ml}$) was added to an aliquot of culture and cells lysed after the indicated times. For lane "0", OXO was added to 1 ml lysis solution to a final concentration of 60 $\mu\text{g/ml}$ and then untreated culture was added. For the negatively numbered lanes, untreated culture was combined with lysis solution and then OXO was added at the indicated times to 30 $\mu\text{g/ml}$. The migration positions of highly negatively supercoiled closed circular, nicked circular, and full-length linear plasmid are indicated by "I", "II", and "III", respectively.

bromide, gave populations with $\bar{\tau}^\circ$ values of -2, -5, -8, -11, -14 and -21, respectively.

RESULTS

Analysis of topoprofiles

The cell lysis procedure, which is described above, was developed to allow as faithful an indication as possible of the topoprofile which existed in the cell at the moment of sampling. A primary concern, that lysis was occurring sufficiently rapidly, was addressed using OXO, a drug known to induce linearization of plasmid when added to DNA gyrase reaction mixtures before terminating the reaction with SDS [22]. Since we used SDS to lyse the cells, we found that OXO causes plasmid linearization *in vivo* as well. The rapidity of cell lysis and inactivation of DNA gyrase could therefore be measured by the speed with which OXO-induced linearization is prevented. Figure 1 shows the effect of addition of lysis solution to cells at various times relative to addition of OXO. Simultaneous addition of OXO and lysis solution led to some linearization. However, 5 sec of exposure of the cells to the lysis solution before OXO addition was sufficient to completely prevent linearization. To test the rapidity of OXO action, the influence of the drug on DNA replication was examined. As shown in Fig. 2, OXO acts within 10 sec to block ^3H -thymidine incorporation. On the basis of these two experiments, we conclude that topoisomerization of plasmid, by DNA gyrase at least,

Figure 2: The kinetics of OXO inhibition of DNA synthesis. ³H-thymidine (80 μ l, 1 mCi/ml, 82 Ci/mmol) was added to 8 ml aliquots of a culture at $A_{450} = 0.12$. cold 5% TCA at various times before (25 μ g/ml) which was added 93 ± 5 sec after addition of H-thymidine. Five independent culture samples were these 5 were normalized to give "1.0" 310,000 to 410,000 cpm. In received no OXO showed ³H-thymidine entire length of labelling (data not samples were filtered onto GFA filter dried, and counted in toluene/PPO.

was blocked more rapidly than 15 sec after addition of lysis solution.

The intensity of the bands using our standard procedure is approximately as expected. A sample of lysate gives a total of about 5 ng of plasmid on an autoradiogram (by comparison with a standard loading of purified plasmid). Knowing the cell density, one calculates an approximate copy number of 100, a value which is reasonably close to that established by other means [23].

Not all *E. coli* strains lyse so quickly. Using turbidimetry as an auxiliary method of measuring the rate of cell lysis, we found that four *E. coli* K12 strains and two *E. coli* B strains lysed far more slowly than AS19 or its derivative DK6recA⁻(pBR322), the strain used in this study. AS19 (a derivative of *E. coli* B) is highly permeable to a spectrum of antibiotics [24] and we see here that it is apparently more fragile as well. Other more refractile strains may be lysed rapidly by using an SDS, EDTA solution at 80° (as opposed to the standard 42°), but the intensity of the bands on the

Figure 3: The topoprofile of plasmid from exponential *E. coli*. Lysate was run either on a standard gel (lane 1) or on a gel containing $6\text{ }\mu\text{g}$ chloroquine per ml (lane 2). Topological marker populations were also run on the chloroquine-containing gel (lane 3: $\tau^{\circ} = -21$, lane 4: $\tau^{\circ} = -2$) and the centers of their distribution are indicated. The position at which $\tau^{\circ} = -21$ migrates in the gel lacking chloroquine is also indicated.

autoradiogram is thereby reduced 5 to 10-fold for some unknown reason. We therefore recommend that, when possible, AS19 and its derivatives be used in conjunction with this lysis procedure.

Using the new technique to analyze pBR322 from exponentially growing cells, we see quite a broad topoprofile. Figure 3 shows a sample run on two gels which allow resolution of different regions of the topoprofile. The highly negatively supercoiled forms are resolved in the rightward gel by addition of chloroquine, an intercalating agent, to the gel buffer. Upon binding to DNA, chloroquine unwinds the primary DNA helix [25] thereby removing negative supercoils or, in the case of molecules of low superhelical density (e.g. $\tau^{\circ} = -2$ in Fig. 3), introducing positive supercoils. Taking into consideration both gels, one estimates that supercoiling extends from about -13 to -32. The distribution is centered around $\tau^{\circ} = -27 \pm 2$, or a superhelical density of $0.064 \pm .005$ supercoils per helical turn.

Influence of NOV on topoprofile

NOV and its analog coumermycin have been shown both *in vivo* [1] and *in vitro* [26] to inhibit the supercoiling activity of DNA gyrase. Fig. 4 shows the time course of the influence of a low NOV concentration on the topoprofile. We anticipated that the drug would merely promote the relaxation of supercoils. However, note the formation of a new ladder of bands as well. At 1 min, only a single ladder is apparent, at 10 min, a second ladder has become equivalent in intensity to the first, and by 30 min, the second ladder predominates. Besides the new ladder of bands, also note that at longer times, hybridizable material appears which migrates faster than the dark band of highly negatively supercoiled species. Since at all NOV concentrations examined, 40 and 60 min of drug treatment gave identical topoprofile (data not shown),

Figure 4: The time course of NOV treatment. NOV ($1 \mu\text{g/ml}$) was added to a culture and samples were lysed at the indicated times (in min). "Ctrl" indicates a sample from an untreated culture. After 30 min of drug treatment, the cells were sedimented, washed in fresh medium, and resuspended in this drug-free medium to their original density. Samples were taken at 1, 3, and 9 min after washing away the drug (1W, 3W, and 9W, respectively).

the endpoint of the drug's action seemed to be reached by 40 min. Fig. 4 also shows that removal of the NOV allows fully negatively supercoiled material to be regenerated.

Electrophoresis in a second dimension in the presence of high chloroquine (Fig. 5) shows not only that the new ladder can be resolved from the old, but also shows the new ladder to be composed of positively supercoiled topoisomers. Indeed, this seemed likely from Fig. 4 since both positively and negatively supercoiled topoisomers are known to migrate on gels faster than relaxed cccDNA. Fig. 5A, a two-dimensional gel of a sample of lysate from a control culture, provides an orientation. The very highly negatively supercoiled species maintained a negatively supercoiled conformation when run in the second dimension. The less highly negatively supercoiled species bound enough chloroquine in the second dimension to adopt a positively supercoiled conformation as seen

Figure 5: Two-dimensional gels of lysates. Samples of lysates from an untreated culture (A) and from a NOV-treated (100 $\mu\text{g}/\text{ml}$, 22 min.) culture (B) were each combined with a mixture of full-length linear plasmid (4360 bp) and 3610, 3260, and 2940 bp pBR322 restriction fragments (denoted by unmarked arrows in A) and run (left to right) in a gel containing 0.5 μg chloroquine per ml. The lanes were excised, soaked in gel running buffer containing 12 μg chloroquine per ml and embedded in place of some of the sample slots of fresh gels containing 12 μg chloroquine per ml. A remaining leftward slot in each gel was loaded with $\tau^{\circ} = -2$ marker. The second dimension was run (top to bottom) and the gels blotted and hybridized all in standard fashion. An unidentified band is denoted by "?".

in the right to left downward curving arc at the top. The five non-closed circular species all migrate on a diagonal, since chloroquine is not capable of altering their conformation. The less intense arc at the top of the gel is an as yet unidentified series of plasmid forms.

Applying this orientation to Fig. 5B, we see precisely the pattern which one would expect if the second ladder was positively supercoiled in the first dimension. All the topoisomers migrate in the second dimension as positively supercoiled species (except for the first dimension's most highly negatively supercoiled forms which, although considerably depleted by NOV, still run as negatives). The topoisomers which comprised the negative ladder in the first dimension now comprise the upper arm of the "<" shape. This arm has the observed slope since the greater the negativity, and therefore the greater the mobility, in the first dimension, the lesser the positivity, and therefore the lesser the mobility, in the second dimension. On the other hand, the positive species in the first dimension should be even more positive in the second dimension and this is the basis for the slope of the lower arm. Note that the lower arm extends further to the right than does the upper arm. This means that the hybridizable material which appeared late in the time course of Fig. 4 is simply an extension of the second ladder and possesses the highest degree of positive supercoiling. As expected, the topoisomers which ran as relaxed forms in the first dimension coincide in mobility in the second dimension with the relaxed marker population of the left lane of Fig. 5B.

Support for the conclusion that positive supercoils are generated by NOV treatment is provided by the differential action of topoisomerase I from *E. coli* (ω protein) on the two ladders (Fig. 6). ω protein is incapable of relaxing topoisomers which either contain positive supercoils [27,28] or which have a low (negative) supercoil density [27,29]. Lane 2 demonstrates that the negatively supercoiled species are relaxed to a high degree, but the second ladder appears unaltered. Even the high mobility material which was shown to be the highly supercoiled extension of the second ladder (Fig. 5B) is unperturbed by the action of ω protein. This rules out the possibility that the enzyme failed to act on this ladder because of insufficient supercoiling of its species. The inability of the enzyme to remove low numbers of negative supercoils can be seen in lane 2 by the augmentation of the less supercoiled region of the negative ladder rather than simply its depletion after ω protein treatment.

Lane 3 demonstrates that both ladders are depleted by treatment with CHO cell nuclear extract. The newly formed species of lane 3 are relaxed topoisomers and not nicked plasmid since their mobility shifts when chloroquine is added to the gel (data not shown). Type I topoisomerase isolated from mammalian nuclei has been shown to be capable of relaxing both positively and negatively supercoiled species [30]. Besides providing a control for lane 2, lane 3 also strongly suggests that the second ladder was

Figure 6: The action of two topoisomerases on plasmid from a NOV-treated culture. Lysate from a NOV-treated (100 $\mu\text{g/ml}$, 6 min) culture was ethanol precipitated (to rid the DNA of residual pronase) and run on the gel after treatment with no enzyme (lane 1), ω protein of *E. coli* (lane 2), or CHO cell nuclear extract (lane 3). The ω protein reaction contained 0.15 μg DNA, 1.5 μg ω protein (to insure that the chromosomal DNA present would not prevent full relaxation) in 10 mM Tris, 6 mM MgCl_2 , 1 mM EDTA. The nuclear extract reaction contained 0.15 μg DNA, 5 μl nuclear extract in 0.17 M KCl, 40 mM Tris HCl pH 7.5 and 1 mM EDTA. Both reactions proceeded for 5 hr at 37°.

composed of topoisomers, a fact which had been presumed until now. The possibility that the second ladder was composed of some sort of series of catenanes rather than topoisomers was eliminated in another experiment in which DNase I was shown to convert both ladders to material with the mobility of nicked plasmid (data not shown).

Fig. 7 examines the NOV dose-response at long times of exposure. Fig. 7A, in which a broad range of NOV concentrations was used, shows that high drug levels inhibit relaxation. To check whether an even more pronounced drug effect might have been missed in Fig. 7A, the dose response in the intermediate range was examined in greater detail (Fig. 7B). Indeed, it appears that even the very optimal NOV doses (80 to 120 $\mu\text{g/ml}$) allowed the maintenance of a sub-population of highly negatively supercoiled plasmid. Coumermycin was examined as well and results were similar to those obtained with NOV (data not shown). Notice that the only indications of the second ladder in Fig. 7 are its resolution from the first in lane 0.3 and the appearance of its extension (which can be seen to be composed of bands in some of the lanes). Whether or not the two ladders are superimposed depends very subtle aspects of the gel running procedure. Thus, a sample which clearly shows a double ladder in one gel (Fig. 4, lane 30) may show only a single ladder in another gel (Fig. 7, lane 1).

Examination of the action of OXO in detail

The effect of OXO was introduced in Fig. 1, but only the drug's linearizing effect was discussed. Fig. 1 also shows that OXO profoundly changed the supercoiling of much of the plasmid which escaped linearization. The double ladder suggestive of positively as

Figure 7: Concentration dependence of prolonged NOV treatment. Culture aliquots were supplemented with the indicated amounts of NOV (in $\mu\text{g/ml}$) and lysed after 60 min (a) or 40 min (b).

well as negatively supercoiled plasmid was also seen after long OXO treatment (Fig. 1, lane +600). A two-dimensional gel confirmed that this second ladder generated by OXO treatment (30 $\mu\text{g/ml}$ 10 min) was indeed positively supercoiled plasmid (data not shown). As shown in the dose response experiment of Fig. 8, OXO produced no detectable shift in supercoiling below 1 $\mu\text{g/ml}$, exerted its optimal effect around 5 to 30 $\mu\text{g/ml}$, and inhibited the shift at 200 $\mu\text{g/ml}$. A two-dimensional gel of the sample of lane 200B confirmed that positive supercoiling as well as relaxation of negative supercoils was inhibited at high OXO levels (data not shown).

As far as the effect of OXO on plasmid linearization is concerned, Fig. 8 shows that the optimal effect was reached in 20 sec when 1 to 200 $\mu\text{g/ml}$ was used. This is in contrast to the slower response of the topoprofile to the drug. OXO appears to be ineffective at 0.01 $\mu\text{g/ml}$, exerts a marginal effect on linearization at 0.1 $\mu\text{g/ml}$, and maximally linearizes plasmid in the range of 1 to 200 $\mu\text{g/ml}$. We see no evidence for the formation of smaller than full-length linear forms even at the highest OXO levels. This is not surprising since more than one non-specific double stranded cut per molecule should not yield discrete bands. However, if multiple cutting were occurring we might

Figure 8: The dependence of linearization and topoprofile alteration on OXO concentration. Cultures were supplemented with various OXO concentrations (indicated in $\mu\text{g/ml}$) and lysed after 20 sec (A) or 10 min (B).

anticipate a depletion of the full-length linear band at high OXO levels, a result which is not observed.

It was conceivable that the positive ladders seen after both NOV and OXO treatments were artifacts arising from the electrophoresis of relaxed plasmid in the presence of NOV or OXO treated cell lysate. This was addressed by the following reconstruction experiment: Plasmid-less DK6recA⁻ was grown and lysed as usual with either NOV treatment (100 $\mu\text{g/ml}$, 30 min.), or OXO treatment (30 $\mu\text{g/ml}$, 10 min.), or no drug treatment prior to lysis. Each lysate (80 μl) was combined with 5 ng of pBR322 which was previously relaxed with Sp2/0 cell nuclear extract, the mixtures run on a gel containing 8 μg chloroquine per ml, and the gel blotted and hybridized. Neither the OXO treated, nor the NOV treated, nor the control lysate had any influence on the mobility of the topoisomers. Therefore, in order for the plasmid to exhibit positive supercoils, it needs to be contained in a viable cell which received NOV or OXO treatment rather than merely be exposed to lysate of such drug-treated cells.

ATP starvation causes plasmid relaxation

A previous study [31] showed that omission of a carbon source and addition of DNP to the growth media depleted ATP by 30-fold after 10 hr. Fig. 9 shows that this protocol promotes substantial plasmid relaxation as well. Although depletion of ATP has not been re-examined in this study, it seems likely that the observed effect of DNP on topoprofile is due to ATP starvation. DNP seems to be specific in its action as an uncoupler of oxidative phosphorylation [32]. Also, the cells rapidly re-introduce supercoils after the addition of glucose back to the culture (lanes 7, 8, 9), correlating with the rapid restoration of ATP levels under the same conditions [31].

One might have anticipated the appearance of positive supercoils after ATP starvation since NOV is known to inhibit DNA gyrase *in vitro* by competitive inhibition of

Figure 9: Alteration of the topoprofile by ATP starvation. The numerical order of the lanes corresponds to the order of lysis of samples from a single 20 ml culture. Lane 1: untreated culture. Lane 2: 10 min after sedimentation and resuspension in minimal media (done twice to insure nutrients are fully removed). At this point, DNP (5 mM) was added to the culture. Lanes 3, 4, 5: 10 min, 70 min, and 7 hr after DNP addition. At this point, cells were twice sedimented and resuspended in fresh minimal media. Lane 6: 5 min after resuspension. At this point, glucose (0.2%) was added. Lanes 7, 8, 9: 2, 12 and 30 min after glucose addition.

ATP binding [26]. However, a two-dimensional gel of the sample from Fig. 9, lane 6 failed to reveal any positive topoisomers (data not shown).

Temperature inactivation of *gyrB* protein also causes plasmid relaxation

A temperature shift experiment was done using LE316(pBR322), a transformant of a strain [13] which is temperature sensitive by virtue of a mutation in *gyrB*. Fig. 10

Figure 10: Alteration of topoprofile due to temperature shift of LE316(pBR322). After growth of the culture at 30° to $A_{450} = 0.13$ and lysis of a sample (lane 1), it was shifted to 42°. Samples were taken 5 min (lane 2), 10 min (lane 3), and 20 min (lane 4) after the shift. The culture was then returned to 30° and the final sample was taken after 15 min (lane 5).

shows the change in topoprofile which accompanied the temperature shift. After shift to the restrictive temperature, a large degree of plasmid relaxation occurred within 5 min. Return of the culture to the permissive temperature completely restored the initial topoprofile within 15 min. A two-dimensional gel of the sample from lane 4 showed no positively supercoiled species (data not shown).

Polyamine starvation exerts no significant influence on topoprofile

It was proposed [33] that the effects of polyamine starvation on *E. coli* physiology (two-fold slowing of the rates of both DNA replication fork movement and RNA elongation) could be mediated by a reduction of DNA supercoiling. The method of topoprofile analysis reported here was originally developed to test this hypothesis. However, no significant change in topoprofile was found when DK6recA⁻(pBR322) was starved for polyamines and lysed by the method presented here (data not shown). Since we were concerned that residual spermidine present in this polyamine auxotroph might mitigate a possible effect on topoprofile, the experiment was also performed on EWH319(pBR322), a complete polyamine auxotroph [14]. Again, no influence of polyamine starvation on topoprofile was detected (data not shown).

DISCUSSION

The new technique for analysis of topoprofiles

When compared with procedures which involve both sedimentation of the cells prior to lysis and purification of plasmid before analysis on ethidium-stained gels, the present procedure has distinct advantages: a) *In situ* lysis is more likely to yield a topoprofile which actually existed at the time of sampling (discussed further below). This is important not only for exponentially growing cultures, but also for experiments in which the cells are exposed to a drug prior to lysis. The sedimentation and resuspension of these cells into buffer which does not contain drug places the cells in undefined conditions prior to lysis. The avoidance of manipulation of cells prior to lysis is also an advantage in experiments in which time points are taken after a change in culture conditions. b) The omission of any purification steps insures that analysis includes all plasmid species present in the cell. c) Being of high sensitivity, this procedure can examine cells from low-density, exponentially growing cultures, i.e., those which have been neither amplified with chloramphenicol nor grown to stationary phase.

The present procedure is capable of accommodating many samples of cells in a single experiment, a technical advantage made possible both by the high sensitivity of autoradiography (thus requiring only small samples of cells to be harvested) and by the ease of preparing each sample. The new procedure also has its disadvantageous aspects: a) the blotting and hybridization procedure is somewhat slow, requiring approximately 6 days and b) the procedure is restricted to *E. coli* B strain AS19. The latter is only a

disadvantage if one must do experiments with mutants which were obtained in *E. coli* K12 (since transduction from *E. coli* K12 to B is often difficult). AS19 is actually advantageous to use in many cases because of its acute drug sensitivity [24].

The major advantage of this new method is that in principle, it is more likely to reveal the *in vivo* pattern of plasmid supercoiling. However, what indications are there that previous procedures were not sufficiently exacting in this regard? Our own experience was with polyamine-starved *E. coli*. When plasmid was purified in a CsCl/EtdBr gradient after lysis of these cells using a conventional technique (sedimentation of exponential cells and lysis using lysozyme, EDTA, and Triton X100), a decrease in plasmid supercoiling was observed upon polyamine starvation [34]. This effect was variable (although always in the same direction), thus compelling us to develop a more reproducible procedure. As stated in Results, the present procedure reproducibly showed no effect of polyamine starvation on topoprofile, suggesting that the old procedure for plasmid analysis did not reflect its true state of affairs in the cell.

We are aware of two other studies which were concerned with topoprofiles of plasmid populations isolated by conventional techniques. Pruss et al. [35] found that pBR322 from wild type *E. coli* had about the same center of supercoil distribution as reported here. Shure et al. [25] found that minicol plasmid had quite a high supercoil density when purified by their procedure. Both studies observed a much tighter distribution of topoisomers than was seen in Fig. 3 of this paper. The broad distribution observed here is not surprising given the replicational and transcriptional activities of the plasmid at the time of lysis.

Positively supercoiled plasmid

We have demonstrated the presence of positively supercoiled plasmid in *E. coli* treated with high levels of either NOV or OXO, representatives of two classes of inhibitors of DNA gyrase. To our knowledge, this is the first demonstration of positive supercoils *in vivo*. This observation was made possible, in part at least, by our use of a drug-permeable strain that is approximately 100-fold more sensitive to NOV than are standard *E. coli* strains [36,37]. Our employment of the new lysis procedure might also have contributed to our ability to observe positive supercoils since the cells were exposed to the necessary high drug doses until the moment they were lysed. Since we have shown that removal of NOV from the media causes depletion of positive supercoils, it is possible that conventional lysis procedures, which involve sedimentation of cells and their resuspension in drug-free buffer, could also allow such dissipation.

Treatment with NOV seems to be more suitable for preparation of large amounts of positively supercoiled DNA for physical and enzymatic studies than are methods which use stoichiometric quantities of purified DNA gyrase [38] or Topo II' [28]. A CsCl, ethidium bromide gradient can easily fractionate the wide range of topoisomers generated

by the present method.

What might be the mechanism of introduction of these positive supercoils? Two parameters of a molecule of cccDNA dictate its degree of supercoiling: a) the number of times the two DNA strands are physically intertwined (the "linking number"), a property that can only be altered by covalent modification and, b) the number of helical turns, a parameter which depends on temperature, ionic strength, intercalator binding, i.e., the environment in which the DNA finds itself. If these two quantities are not equal, the molecule must take on supercoils to resolve the discrepancy. The handedness of the supercoils will depend on the relative size of the two quantities: a paucity in linking number relative to helical turns will produce "underwound" or "negative" supercoils; "positive" or "overwound" supercoils arise when the linking number is higher than the number of helical turns. It follows that if a negatively supercoiled molecule were to be melted (i.e., if some of the helical turns were to be removed), the supercoils would be dissipated. However, if one melts a positively supercoiled molecule, the discrepancy between the two parameters would only be augmented thus forcing this molecule to assume an even more highly supercoiled conformation. Since a major physiological role of supercoiling is probably to facilitate melting during replication and transcription, the reason for the existence of negative rather than positive supercoils *in vivo* is apparent.

But the superhelical torsion observed upon analysis of DNA *in vitro* did not necessarily have to be present as such *in vivo*. For example, eukaryotic viruses [39], and perhaps eukaryotic DNA in general [40], seem to "store" all their negative supercoils in solenoid-type structures (nucleosomes). For bacterial DNA *in vivo*, it seems that approximately half the negative supercoils are thus restrained [40,41]. We suggest that the positive supercoils which are observed after treatment of *E. coli* with DNA gyrase inhibitors may be another example of restrained supercoils. Furthermore, DNA gyrase itself may be the restraining agent.

Formally, there are three ways that alteration of DNA structure *in vivo* induced by gyrase inhibitors could give rise to positive supercoils: 1) The drugs could somehow induce the cells to increase the (primary) helical twist of the DNA. Relaxation *in vivo*, followed by cell lysis to remove the agent which increases the helical twist, would then give rise to positive supercoils. We have evidence that both NOV and OXO are incapable of such a structural alteration: the end-point of relaxation of pBR322 by Sp2/0 cell nuclear extract is unaffected by concentrations of NOV and OXO up to 0.3 mg per ml and 0.2 mg per ml, respectively (using the relaxation reaction conditions described in "Methods"; unpublished results). The fact that agents which increase DNA helical twist are quite rare (netropsin is one such ligand [J. C. Wang, personal communication]) argues against, although does not exclude, the possibility that OXO or NOV addition to

the culture somehow activates such an agent. 2) It is conceivable that there are actually unrestrained positive supercoils in the DNA of drug-treated cells. One would have to argue for the existence of some "anti-gyrase" activity which could generate these. Even if NOV or OXO could induce such an activity, it seems likely that Topoisomerase II' would simply dissipate these supercoils since it is known to relax positive supercoils at least *in vitro* [28]. However, one cannot fully exclude this unlikely possibility at present. 3) The positive supercoils could be restrained in solenoid-type structures in the cell. As mentioned above, DNA gyrase could be the core of such a structure. DNA binds to DNA gyrase by wrapping around the enzyme [42]. This wrap is equivalent in bondedness to positive supercoiling [38]. It is conceivable that some entity other than gyrase produces such a solenoid, but no other such agent (from any organism) is presently known.

We suggest that NOV and OXO induce the formation of positive supercoils as a direct result of their inhibition of the supercoiling activity of DNA gyrase. Thus, upon drug addition, the DNA undergoes relaxation due to the relaxation activity either of gyrase, of some other topoisomerase, or some combination thereof. From *in vitro* studies, it is clear that DNA gyrase has a more pronounced affinity for relaxed than for negatively supercoiled DNA [43,44]. Relaxation would thus promote the additional binding of gyrase, thereby introducing positive, restrained supercoils and compensating tense negative supercoils in the DNA. The latter then would be relaxed by the same agent that was responsible for the initial relaxation. This process of gyrase binding and DNA relaxation would continue until an equilibrium is reached at which the unused pool of gyrase is mostly depleted. Lysis of the cells would remove the bound gyrase and convert the restrained positive supercoils into tense ones.

OXO-induced linearization

OXO, in addition to altering the topoprofile, is capable of inducing plasmid linearization when cells are lysed by the present procedure. But even at high OXO levels, only a fraction of total plasmid is linearized. One explanation for this might be that a portion of the OXO-induced covalent gyrase-DNA complexes is destroyed by the EDTA contained in the standard lysis solution before SDS is capable of denaturing them. DNA gyrase reaction mixtures inhibited by OXO respond in this way to EDTA prior to SDS [44]. However, no change in degree of OXO-induced linearization was seen when cells were lysed with only SDS and supplemented with EDTA 30 sec later, thus disfavoring this explanation (data not shown). Another explanation of the fractional plasmid linearization, that OXO itself is unable to interact with more than 30% of the plasmid, is unlikely since the highest OXO level (200 $\mu\text{g/ml}$), although it is capable of inhibiting relaxation and is therefore exerting more of an effect on cells than are lower drug levels, fails to induce further linearization.

It is possible to account for this result by a model in which a portion of the plasmid population bears no DNA gyrase and is therefore not susceptible to the action of OXO. Another observation also points toward such a model: the persistence of a sub-population of cccDNA which remains highly negatively supercoiled even at optimal NOV or OXO levels. Since Topo I (ω protein) seems capable of relaxing DNA *in vivo* [6,35], this sub-population of plasmid would need to be sequestered from this enzyme as well for the observed high number of negative supercoils to be retained. It is conceivable that the cell controls supercoiling and thereby various processes dependent on it by controlling the accessibility of topoisomerases to DNA in some way. Our evidence is consistent with limited access, but it does not address the question of possible alteration of this accessibility under different growth states.

OXO-induced linearization *in vivo* allows identification of the sites of gyrase interaction with plasmid in the cell. *In vitro* studies [10,11,12] have shown that gyrase binds to specific sites on DNA but a comparison of these sites fails to demonstrate any obvious determinant for site specificity. We are currently mapping the *in vivo* gyrase sites of pBR322 in an attempt to elucidate these determinants.

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* Abbreviations: NOV, novobiocin; OXO, oxolinic acid; DNP, 2,4-dinitrophenol; cccDNA, covalently closed circular DNA; SSC, standard saline citrate (0.15 M NaCl, 15 mM sodium citrate).

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