Novobiocin induces positive supercoiling of small plasmids from halophilic archaebacteria in vivo

M.Sioud, G.Baldacci, A.M.de Recondo and P.Forterre

Laboratoire de Biologie Moléculaire de la Réplication ER 272, CNRS, BP no.8, 94802 Villejuif Cédex, France

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ABSTRACT

The halophilic archaebacterium Halobacterium strain GRB harbours a multicopy plasmid of 1.7 kb which is negatively supercoiled. After addition of novobiocin to culture medium all 1.7 kb plasmid molecules become positively supercoiled. Positive supercoiling occurs at the same dose of novobiocin inhibiting the eubacterial DNA gyrase in vitro. Novobiocin also induces positive supercoiling of pHV2, a 6.3 kb plasmid from Halobacterium volcanii. These results indicate the existence of a mechanism producing positive superturns in halobacteria. The 1.7 kb plasmid from Halobacterium GRB could be used to produce high amounts of pure positively supercoiled DNA for biophysical and biochemical studies.

INTRODUCTION

Superhelicity is an essential feature of DNA shaping and determines a variety of biological events (for reviews, see ref. 1-3). Circular DNA duplexes isolated from cells and viruses are negatively supercoiled. Negative supercoiling facilitates DNA melting whereas fine adjustments of the level of supercoiling may control the strength of DNA-protein interactions.

In contrast to negative supercoiling, positive supercoiling might be unfavourable to replication, transcription and recombination by increasing the stability of the DNA double helix. As expected from such considerations, no positively supercoiled DNA has been isolated from cells in physiological conditions. Therefore, it came as a surprise that the thermophilic archaebacterium Sulfolobus acidocaldarius contains an ATP-dependent type I DNA topoisomerase which introduces positive superturns in covalently closed circular DNA.
DNA: reverse gyrase (4-6). In addition, the DNA of a virus-like particle (SSVI) discovered in Sulfolobus solfataricus B12 is positively supercoiled (7,8).

The archaebacteria are a group of prokaryotes including extreme thermophiles, methanogens and extreme halophiles (halobacteria) (9). The halobacteria are mesophilic organisms which require 3-4 M NaCl in their culture medium and contain 3-4 M KCl in their cytoplasm (10). In contrast to the DNA of Sulfolobus virus-like particle SSVI, the 1.7 kb plasmid of the halophilic archaebacterium Halobacterium GRB is negatively supercoiled (11). Halobacteria probably contain a classical type II DNA topoisomerase since they are sensitive to inhibitors of these enzymes such as the epipodophyllotoxins (VP 16 and VM26) and the coumarines (novobiocin and relatives) (8,11-13). In particular, halobacteria are inhibited by novobiocin concentrations which are otherwise specific for the inhibition of negative supercoiling generated by eubacterial type II DNA topoisomerase (DNA gyrase).

It has been suggested that positive supercoiling by reverse gyrase could prevent the genomic DNA of Sulfolobus from denaturation at high temperature (4). In this hypothesis, positive supercoiling could be restricted to thermophilic archaebacteria. Nevertheless, we report here that novobiocin induces positive supercoiling of small plasmids in halobacteria. The different mechanisms which could produce positive supercoiling in these mesophilic archaebacteria are discussed.

MATERIALS AND METHODS

Bacterial strains, growth and novobiocin treatment.
Halobacterium strain GRB (14) (a gift from U. Rdest, Würzburg University, FRG) and H. volcanii (a gift from I. Rosenshein, Tel Aviv University) were grown at 37°C in liquid shaken cultures. Halobacterium GRB was grown in classical halophile medium (12) and H. volcanii was grown in the medium of Mullakhanbhai and Larsen (15). Novobiocin was added when the cultures reached an optical density of 0.3 at 600 nm.
Enzymes and chemicals.

Ribonuclease, proteinase K, novobiocin and anisomycin were obtained from Sigma (St. Louis, USA), DNAse I from Boehringer (Mannheim, FRG) and restriction enzyme SauI from Gibco BRL (Cergy-Pontoise, France). Rat liver DNA topoisomerase I was a gift of G. Mirambeau (University Paris 6) and netropsin was a gift from C. Zimmer (Iena, DDR).

Purification of the 1.7 kb plasmid from Halobacterium GRB.

Control and novobiocin-treated cells from 500 ml cultures were harvested 18 hours after drug addition and washed in basal salt medium (4 M NaCl, 0.12 M MgSO$_4$, 0.03 M KCl and 0.01 M trisodium citrate, pH 7.2). Cells were lysed in 10 ml of 25 mM Tris HCl, pH 7, 10 mM EDTA. After five minutes at room temperature, 20 ml of an alkaline solution (0.2 M NaOH, 1 % SDS) were added, followed by addition of 15 ml of 3.3 M potassium acetate, pH 4.8. Most of the chromosomal DNA and cell debris were removed by centrifugation at 20,000 rpm for 20 minutes (Sorvall centrifuge, SS34 rotor). The plasmids were precipitated by 0.6 volumes isopropanol. After 15 minutes at room temperature, DNA was recovered by centrifugation, resuspended in 10 mM Tris-HCl pH 7, 1 mM EDTA and purified by ethidium bromide-CsCl gradient centrifugation.

Gel electrophoresis, blotting and hybridization.

DNA samples were run through a 1.2 % agarose gel in TBE buffer at 3 v/cm for 16 hours. In the case of minigels, the electrophoresis was performed at 5 v/cm for 5 hours. For two-dimensional gel electrophoresis, the gels were soaked in TBE buffer containing 0.01 µg/ml ethidium bromide or 12 µM netropsin for 2 hours at room temperature and the second dimension was run in the same buffer for 6 hours. The gels were stained with ethidium bromide and photographed under UV light. Gels containing netropsin were washed 3 times for one hour in distilled water before staining with ethidium bromide. The transfer of DNA onto nitrocellulose, prehybridization, hybridization and washes were performed according to Maniatis et al. (16). The 1.7 kb plasmid probe was prepared using purified plasmid electroeluted from agarose gel and labelled by nick translation.
Preparation of nicked and linearized forms of the 1.7 kb plasmid.

To prepare the 1.7 plasmid form II, the purified 1.7 kb plasmid DNA was treated with DNAse I (1 ng/ml) for 10 minutes at 30°C in 10 mM Tris-HCl, pH 8, 5 mM MgCl₂. The reaction was stopped by addition of EDTA to 10 μM. The reaction mixture was then electrophoresed on agarose gel and the form II was electroeluted. To prepare the form III, the 1.7 kb plasmid was linearized with the restriction endonuclease SalI which has a single cutting site per molecule.

Sedimentation velocity analysis

Purified plasmid DNA was layered over a 10-30% linear glycerol gradient (11.5 ml) performed in a buffer solution containing 3 M KCl, 0.1 M MgCl₂, and 10 mM Tris HCl, pH 7.5. Centrifugation was performed at 36,000 rpm for 18 hours in a SW41 rotor.

RESULTS

Novobiocin induces positive supercoiling of the 1.7 kb plasmid from Halobacterium GRB.

We have used the 1.7 kb plasmid of Halobacterium GRB (1000 copies per cell) as a target to study the response of halobacteria to type II DNA topoisomerase inhibitors (11,13). We reported that the population of 1.7 kb plasmids isolated from VP16-treated Halobacterium GRB cells contained both cleaved molecules with proteins linked to the 5' DNA ends and partially relaxed molecules composed of a mixture of positively and negatively supercoiled topoisomers (13). These topoisomers with a different sign of superhelicity formed a double staggered ladder after agarose gel electrophoresis. In contrast, a single ladder of topoisomers was observed after novobiocin treatment (11). We noticed that this ladder migrated as the positively supercoiled topoisomers produced by VP16; this suggested that novobiocin treatment produces positively supercoiling of all plasmid DNA molecules. To test this hypothesis, we have compared the electrophoretic mobility of purified 1.7 kb plasmid from cells treated or not treated with novobiocin (1.5 μg/ml for 18 hours) in gels containing either
Fig. 1 Topological analysis of purified 1.7 kb plasmid DNA isolated from control and novobiocin-treated cells. Panels A: lane 1, plasmids extracted from untreated cells; lanes 2, plasmids extracted from novobiocin-treated cells. Panel B: ethidium bromide (0.04 µg/ml) was included in the gel and in the electrophoresis buffer: lanes 3 and 4, plasmids from untreated cells; lanes 5 and 6, plasmids from novobiocin-treated cells. Panel C: plasmids extracted from novobiocin-treated cells before (lane 7) and after (lane 8) treatment with rat liver type I DNA topoisomerase as described elsewhere (7). Panel D: netropsin (12 µM) was included in the electrophoresis buffer: lane 9, plasmids from untreated cells, lane 10, plasmids from novobiocin-treated cells.

Ethidium bromide or netropsine. Ethidium bromide is an intercalating dye untwisting the DNA double helix which relaxes negatively supercoiled DNA. In contrast, netropsin, a DNA binding drug, increases the twist of the double helix and relaxes positively supercoiled DNA (17).

Comparison of lane 1 with lanes 3 and 4 in Figure 1 shows that the electrophoretic mobility of the 1.7 kb plasmids isolated from untreated cells decreases in agarose gels containing 0.04 µg/ml of ethidium bromide resulting in the appearance of separated topoisomers. This indicates that the 1.7 kb plasmid of Halobacterium GRB is negatively supercoiled in physiological conditions. Lane 2 of figure 1 shows that the 1.7 kb plasmid isolated from novobiocin-treated cells is composed of topoisomers which are separated in agarose gels. Comparison of lane 2 with lanes 5 and 6 shows that ethidium bromide increases the electrophoretic mobility of these topoisomers.
Fig. 2 Analysis of the 1.7 kb plasmid DNA from novobiocin treated cells by two-dimensional agarose gel electrophoresis. Panel A: plasmid DNA from cells treated with novobiocin for one hour was electrophoresed in a gel containing 0.01 µg/ml of ethidium bromide in the second dimension. Panel B: plasmid DNA from cells treated with novobiocin for 15 hours was electrophoresed in a gel containing 0.01 µg/ml of ethidium bromide in the second dimension. Panel C: plasmid DNA from cells treated with novobiocin for 18 hours was electrophoresed in a gel containing 12 µM netropsin in the second dimension. The arrow indicates the position of form II (migrating as relaxed DNA in the second dimension).

which become combined into a single band. This suggests that the 1.7 kb plasmid molecules extracted from novobiocin-treated cells are composed of either knotted or positively supercoiled molecules. The first possibility is ruled out as this plasmid is relaxed by rat liver type I DNA topoisomerase, an enzyme which cannot unknot duplex DNA (Figure 1, lane 8). Evidence for positive supercoiled DNA is obtained from gels containing netropsin. Figure 1 shows that plasmids from novobiocin-treated cells are relaxed by 12 µM netropsin (compare lanes 2, 7 and 10) whereas migration of plasmids isolated from control cells is unchanged (compare lanes 1 and 9).

Two-dimensional gel analysis was used to further confirm the novobiocin-induced positive supercoiling of the 1.7 kb plasmid. In figure 2, panels A and B, the second dimension electrophoresis was performed in the presence of ethidium bromide (0.01 µg/ml). In this condition, the migration of negatively supercoiled DNA is reduced whereas the migration of positively supercoiled DNA is increased. Panel A shows that 1.7 kb plasmid molecules isolated from cells treated with novobiocin for one hour are composed of a mixture of negatively
supercoiled topoisomers (left branch of the arch) and positively supercoiled topoisomers (right branch). In contrast, all detectable plasmid molecules isolated after 18 hours treatment with novobiocin (panel B) are positively supercoiled (only the right branch of the arch is visible). As expected, this pattern of migration was reversed when 10 \( \mu \)M netropsin was substituted to ethidium bromide in the second dimension (panel C).

The density of positive supercoiling was maximal after 4 hours of incubation with novobiocin and remained constant upon longer time of drug treatment. This positive superhelical density was determined by densitometric analysis to be 0.035 ± 0.005: this corresponds to an average of 5-6 superturns. The topoisomers with 7 or more superturns were not resolved in the agarose gels. It is interesting to note that topoisomers with 6 and 7 positive superturns migrated more rapidly than the native form I which comprises topoisomers with higher superhelical density (up to 11 negative superturns, data not shown). Positive supercoiling was induced by novobiocin concentrations as low as 0.05 \( \mu \)g/ml (not shown). These concentrations are similar to those which specifically inhibit eubacterial DNA gyrase in vitro.

When novobiocin-treated cells were harvested and incubated for two hours in drug-free medium, all 1.7 kb plasmid molecules were fully negatively supercoiled again. This indicates that both negative and positive supercoiling of the 1.7 kb plasmid occur in vivo and suggests that positively supercoiled molecules are substrate of a DNA gyrase-like enzyme in halobacteria. Salt-induced positive supercoiling.

Our finding that 1.7 kb plasmid DNA isolated from novobiocin-treated cells is positively supercoiled after cell lysis does not necessarily mean that this plasmid is positively supercoiled in vivo. In particular, the 1.7 kb plasmid could be relaxed in vivo and positive superturns introduced by the reduction in salt concentration during transfer from the halobacterial cytoplasm (3M KCl, 0.1 M MgCl\(_2\)) to the electrophoresis buffer (the salt effects on DNA superhelicity
Fig. 3 Sedimentation velocity analysis of 1.7 kb plasmids DNA from untreated (A) and novobiocin-treated cells (B) in salt glycerol gradients containing 3M KCl and 0.1 M MgCl2. CsCl-purified plasmid DNA was layered over a 10-30 % linear glycerol gradient. After centrifugation, fractions (0.4 ml) were collected from the top of the gradients; 30 ul of each fraction were electrophoresed in a 1.2 % agarose minigel, transferred to nitrocellulose and hybridized with labelled 1.7 kb plasmid. Panel A: 1.7 kb plasmid from untreated cells mixed with 1.7 kb plasmid DNA form II. Panel B: plasmid from novobiocin-treated cells (1.5 µg/ml for 18 hours) mixed with forms II and III.

are reviewed in ref. 18). To test this hypothesis, we compared the sedimentation pattern of 1.7 kb plasmids isolated from novobiocin-treated and untreated cells (Figure 3, panels A and B, respectively) in glycerol gradients containing 3M KCl and 0.1 M MgCl2. If the positive supercoiling induced by novobiocin is entirely due to the salt effect discussed above, plasmid DNA should sediment in such gradients as relaxed DNA (at the position of nicked DNA). Figure 3 shows that this is not the case: the majority of the positively supercoiled plasmids migrates at the same position as negative form I from untreated cells. However, the salt effect can explain some positive supercoiling since Figure 3 shows that topoisomers +2 and +3 of plasmids isolated from novobiocin-treated cells sediment at the same position as nicked DNA. The position of form III (linear 1.7 kb plasmid) allows to identify the writhing number of the 1.7 kb plasmid topoisomers in panel B as the form III migrates.
Fig. 4 Analysis of the pHV2 plasmids DNA from novobiocin-treated cells in agarose gel electrophoresis. After novobiocin treatment (2µg/ml), the cells were harvested and lysed by addition of TE buffer. Plasmids were isolated by the rapid small scale isolation method described by Maniatis et al. (15). After RNase treatment, the DNA was electrophoresed in 0.8 % agarose gel, transferred to nitrocellulose and hybridized with a labelled pHV2 probe. Panel A: left lane, plasmids isolated from untreated cells; right lane, plasmids isolated from novobiocin-treated cells. Panel B: plasmids isolated from novobiocin treated cells electrophoresed in second dimensions in the presence of 0.03 µg/ml of ethidium bromide. The arrow indicates the direction of migration in the second dimension.

near the topoisomer +2 in our electrophoresis conditions (not shown).

Positive supercoiling of the plasmid pHV2 from H. volcanii.

The species H. volcanii contains a plasmid of 6.3 kb (pHV2) present in approximately 2-3 copies per cell (C.J. Daniels, personal communication). Topological analysis in agarose gels with and without ethidium bromide shows that pHV2 is negatively supercoiled (not shown). Figure 4 compares the electrophoretic mobilities of pHV2 DNA isolated from cells treated or not treated with novobiocin. The two-dimensional analysis shown in panel B clearly demonstrates that pHV2 is positively supercoiled after novobiocin treatment (2 µg/ml). This result
strongly suggests that positive supercoiling after novobiocin treatment is a general feature of small halobacterial plasmids independently of their copy number.

DISCUSSION

Our results show that small plasmids of two halobacterial species are positively supercoiled after treatment with novobiocin at doses which specifically inhibit the eubacterial DNA gyrase. These data indicate that the inhibition of an halophilic gyrase-like enzyme by novobiocin unmasks a DNA topoisomerase activity which increases the DNA linking number. This observation strongly suggests that DNA superhelical density in halobacteria is controlled, as in eubacteria, by a balance between antagonistic DNA topoisomerase activities.

All the detectable plasmid molecules are positively supercoiled after novobiocin treatment. In contrast, we have previously shown that only a small number of 1.7 kb plasmid molecules were positively supercoiled after treatment by VP 16, another type II DNA topoisomerase inhibitor (14). This difference can be explained by an inhibition of the mechanism responsible for positive supercoiling either by VP16 itself or by cleavable complexes induced by this drug.

The discovery of novobiocin-induced positive supercoiling in halobacteria raises two questions: i) does positive supercoiling of halobacterial plasmids occur in vivo? ii) what is the mechanism which creates positive superturns?

The existence and the extent of positive supercoiling in vivo cannot be easily evaluated because the intracellular DNA topology is influenced by DNA binding proteins and environmental effects. In the case of halobacteria, a trivial explanation for the electrophoretic detection of positive supercoiling may be the difference in salt concentration between the cytoplasm and the electrophoresis buffer. We have found that this difference introduces 2-3 positive superturns in vitro in the case of the 1.7 kb plasmid from *Halobacterium GRB* (Fig. 3); on the contrary, the decrease of temperature between the growth medium (37°C) and the agarose gel (20°C) should eliminate about 1 positive superturn in this plasmid,
according to the dependence between temperature and superhelicity (19). Therefore, positive supercoiling in halobacteria cannot be due solely to a salt effect. On the contrary, since the positive form I of the 1.7 kb plasmid DNA isolated from novobiocin-treated cells contains an average of 5-6 positive superturns (see Figures 1 and 2), it should contain at least 3-5 positive superturns in vivo. However, the actual number of free positive superturns in living cells could be higher if DNA-binding proteins prevent the salt-induced winding of the double helix.

The discovery of positive supercoiling of plasmid DNA in halophilic archaeabacteria fits well with the existence of a reverse gyrase in the archaeabacterium S. acidocaldarius and with the positive supercoiling of the SSV1 viral genome. Since reverse gyrase is a type I DNA topoisomerase (5,6), it is tempting to imagine that novobiocin (a type II DNA topoisomerase inhibitor) un masks the activity of an halobacterial type I DNA topoisomerase with reverse gyrase activity. Unfortunately, we have failed up to now to detect topoisomerase activities in crude extracts of halobacteria. This may be due to the difficulty of testing the activity of DNA metabolizing enzymes in the presence of extremely high salt concentration. Consequently, it should be kept in mind that other mechanisms may explain novobiocin-induced positive supercoiling in halobacteria. For example, DNA-binding proteins could wrap in vivo plasmid DNA in a positive sense or else decrease the DNA double helical pitch. Removal of such proteins during cell lysis would result in positive supercoiling of plasmid molecules previously relaxed by a DNA topoisomerase resistant to novobiocin. Loskshon and Morris suggest such a model (wrapping of the DNA in a positive sense around drug-inactivated DNA gyrase molecules) to explain the production of positively supercoiled plasmid pBR322 after novobiocin treatment of Escherichia coli (20). It should be noticed that only some pBR322 molecules are positively supercoiled after novobiocin treatment whereas the drug induces positive supercoiling of all detectable molecules in the 1.7 kb or pHV2 plasmid populations (see Figures 2 and 4). Recently, Liu and
Wang have suggested that positive supercoiling of a circular plasmid may be produced in vivo by RNA polymerases moving in opposite direction when the DNA topoisomerase which normally removes this type of supercoiling is inhibited (21). This mechanism cannot explain positive supercoiling of the 1.7 kb plasmid since we detect only one transcript of this plasmid in Halobacterium GRB cells with or without novobiocin treatment (unpublished data).

The discovery of positive supercoiling in archaeabacteria raises the question of the biological meaning of this DNA structure and stresses the importance to study the biological and biophysical properties of positively supercoiled DNA. Several methods used to prepare such DNA in vitro require a DNA topoisomerase (reviewed in ref. 4), consequently they are not suitable to prepare high amounts of material. On the contrary, the high copy number and the homogeneity of positively supercoiled 1.7 kb plasmids DNA isolated from novobiocin-treated halobacteria make these microorganisms the most convenient source of pure positively supercoiled DNA.

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