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Plasmid pGS5 from the Hyperthermophilic Archaeon *Archaeoglobus profundus* Is Negatively Supercoiled

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We present evidence that, in contrast to plasmids from other hyperthermophilic archaea, which are in the relaxed to positively supercoiled state, plasmid pGS5 (2.8 kb) from *Archaeoglobus profundus* is negatively supercoiled. This might be due to the presence of a gyrase introducing negative supercoils, since gyrase genes are present in the genome of its close relative *A. fulgidus*, and suggests that gyrase activity predominates over reverse gyrase whenever the two topoisomerases coexist in cells.

Previous topological analyses of native plasmids from hyperthermophiles belonging to the two major phylogenetic lineages within the domain *Archaea* revealed a relaxed to positively supercoiled state of DNA (1, 2, 10). This was in contrast to the negatively supercoiled state of DNA in all mesophiles, including mesophilic archaea (1). The strains investigated in those studies were members of the orders *Sulfolobales* and *Thermococcales*, representing the two major archaeal kingdoms, *Crenarchaeota* and *Euryarchaeota*, respectively. All of them possessed reverse gyrase, a topoisomerase specific for hyperthermophiles that is able to introduce positive DNA supercoils (5). Therefore, reverse gyrase appeared responsible for this peculiar topological state. Accordingly, the idea that DNA was stabilized in organisms living at very high temperatures by an overall linking excess seemed to be supported (6). However, this hypothesis was called into question when plasmid pRQ7 from the hyperthermophilic bacterium *Thermotoga maritima* was found to be negatively supercoiled (7). In addition to reverse gyrase, *T. maritima* possesses DNA gyrase, a typical bacterial topoisomerase that introduces negative supercoils into DNA and is responsible for the negative supercoiling of pRQ7 (7). Two alternative possibilities could be then considered to explain the different plasmid topologies in hyperthermophiles. Either gyrase activity dominates whenever both reverse gyrase and gyrase are present in the cell, maintaining an overall DNA negative supercoiling, or, alternatively, the relaxed to positively supercoiled plasmid topology could be a taxonomic characteristic specific to hyperthermophilic archaea. The discovery of pGS5, a plasmid of 2,802 bp in the sulfate-reducing hyperthermophilic archaeon *Archaeoglobus profundus* (G. Erauso et al., unpublished data), allowed us to test which possibility was correct since the complete genome sequence of the closely related species *Archaeoglobus fulgidus* revealed the existence of genes encoding both gyrase and reverse gyrase (9).

A. profundus strain AV18 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM 5631) and cultivated under strict anaerobic conditions in a standard medium (Erauso et al., unpublished) consisting of a basal salt solution (containing, per liter, 20 g of NaCl, 3 g of $MgCl_2 \cdot 6H_2O$, 4 g of Na_2SO_4, 0.5 g of KCl, 0.25 g of NH_4Cl, 0.15 g of $CaCl_2 \cdot 2H_2O$, and 0.14 g of K_2HPO_4) plus 1 ml of a trace element solution (containing, per liter, 100 mg of $Na_2WO_4 \cdot 2H_2O$ and 100 mg of $NaSeO_3 \cdot 2H_2O$) buffered to pH 6.8 with 4 g of piperazine-*N*-*N*′-bis(2-ethanesulfonic acid) (PIPES) sodium salt and supplemented with 1 g of yeast extract (Difco) and 4 g of sodium acetate. Resazurine (1 mg $liter^{-1}$) was used as a redox indicator, and $Na_2S \cdot 9H_2O$ was used as a reducing agent at a final concentration of 0.05% (wt/vol). Cultures (100 ml) were grown in 250-ml sealed bottles pressurized with H_2 and CO_2 (80:20, 200 kPa) and placed in a shaking incubator at 130 rpm and 80°C, the optimal growing temperature of *A. profundus*. Cell growth was monitored by direct cell counting using a Bürker-Türk counting chamber (depth, 0.01 mm). Growth was stopped at the late exponential phase either by allowing the culture to cool slowly to room temperature or after immediate chilling on ice to avoid any topological changes induced by residual topoisomerase activities during the process (10, 12). Cells were collected by centrifugation at low speed, pellets were resuspended in 0.8 ml of ice-cold HNE buffer (50 mM HEPES, 350 mM NaCl, 1 mM EDTA [pH 7.0]), and plasmid DNA was extracted by an isothiocyanate-phenol extraction method as previously described (10). Plasmid topoisomers were then fractionated by electrophoresis in 1% agarose gels prepared in TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8]). Preliminary electrophoretic analysis seemed to indicate that pGS5 was negatively supercoiled. Therefore, we added the intercalating drug chloroquine to a final concentration of 2 μg/ml from a freshly prepared 20-mg/ml solution to allow topoisomer resolution in subsequent electrophoreses. These were performed at 25°C and 20 mA for 20 h in TBE containing chloroquine, and the gels were extensively washed with water to eliminate part of the chloroquine prior to staining with ethidium bromide (1 μg/ml). Stained gels were photographed under UV light using the SONY UVP Image Store 5000 system, and images were stored using Adobe Photoshop 5.0.

pGS5 topoisomers were found to be negatively supercoiled by comparison to topoisomers from control negatively supercoiled and relaxed plasmids of similar size (Fig. 1). Chloroquine intercalates in DNA in such a way that negatively supercoiled topoisomers become more relaxed. Thus, they can be separated as single bands, instead of a single-front band as in

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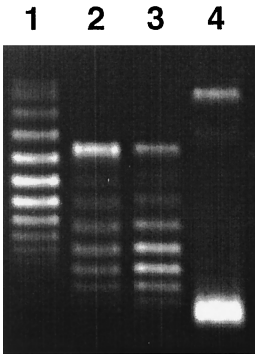

FIG. 1. Resolution of pGS5 topoisomers by one-dimensional gel electrophoresis in the presence of 2 μg of chloroquine per ml. Samples correspond to negatively supercoiled pTZ18 (2,880 bp) (lane 1), pGS5 extracted from cells allowed to cool slowly (lane 2) or immediately chilled (lane 3), and pTZ18 relaxed at 25°C that, under these conditions, migrates as a single band of positively supercoiled topoisomers (lane 4).

normal gels, due to reduced electrophoretic mobility (Fig. 1, lane 1). Similarly, relaxed topoisomers become positively supercoiled and migrate faster, generating a single-front band under the conditions we used (lane 4). However, in one-dimensional gels, topoisomers with an identical number of positive and negative supercoils (+1 and −1, +2 and −2, etc.) comigrate. Therefore, although comparison with control plasmids indicated that pGS5 was negatively supercoiled, we carried out two-dimensional electrophoresis with a higher concentration of intercalating agent added in the second dimension. This method allows unambiguous resolution of negative topoisomers, which become relaxed and migrate slowly, from positive topoisomers, which become more positive (hence more compact) and migrate faster. For the first dimension, samples were electrophoresed at 20 mA for 20 h in the presence of chloroquine (2 μg/ml). After equilibration of the gel in TBE containing 5 μg of chloroquine per ml, the second dimension was run in the same buffer at 15 mA for 20 h. As can be seen in Fig. 2, pGS5 topoisomers are negatively supercoiled, since they remain on the left of an ideal arc that would be formed if all possible topoisomers were present. Positive topoisomers would occupy the right portion of that ideal arc.

The most intense pGS5 topoisomer was −4 under the conditions used. The average specific linking difference (σ) for pGS5 was then determined using the equation $\sigma = \Delta Lk/Lk_0$, where ΔLk (Lk is the plasmid linking number) corresponded to −4 by the band-counting method (8), with a relative precision of ±0.5. To account for the effect of the chloroquine added to the gels, we deduced the expected pGS5-specific linking difference at 25°C by extrapolation after comparison with the bacterial plasmid pTZ18, whose superhelical density is known (σ = −0.052 at 25°C) (Fig. 1) (2). The σ value calculated for pGS5 in this way was then corrected to take into account the difference between the temperature of the gel electrophoresis (25°C) and the temperature at which the cultures had been grown (80°C). For this, we used the value −0.011°/degree Celsius/base pair as an estimation of the rotation angle of the DNA double helix with temperature (2–4), which allowed us to determine a specific linking difference for pGS5 of −0.033 ± 0.005 at the optimal growth temperature of *A. profundus*.

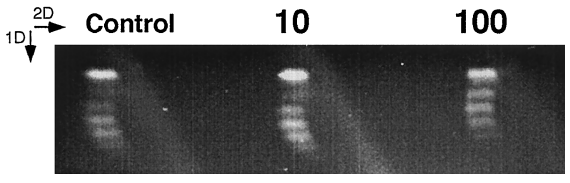

FIG. 2. Two-dimensional gel electrophoresis of pGS5 (2,802 bp) extracted from cells treated with 0, 10, or 100 μg of novobiocin per ml, as indicated above each lane. Chloroquine (2 and 5 μg/ml) was used during the first and second dimensions, respectively.

Interestingly, the same extent of negative supercoiling was observed in pGS5 when the cultures had been cooled rapidly or allowed to cool slowly to room temperature (Fig. 1, lanes 2 and 3). By contrast, in other hyperthermophilic archaea devoid of gyrase, *Lk* decreases rapidly when cultures are chilled slowly, which somehow mimics the effects of a cold shock (10, 12).

The finding that pGS5 is negatively supercoiled in *A. profundus* recalls the situation in the bacterium *T. maritima*, where both gyrase and reverse gyrase are present but where the gyrase activity predominates and the plasmid DNA is globally negatively supercoiled (7). The small pRQ7 plasmid (846 bp) was found to have a specific linking difference of −0.049 ± 0.005 at 80°C, moderately more negatively supercoiled than pGS5 (−0.033 ± 0.005) at the same temperature. Since σ is a relative measure of ΔLk independent of plasmid size, the two values can be readily compared. These results indicate that negative DNA supercoiling can also be found in hyperthermophilic archaea under normal conditions and that this is possibly linked to the presence of gyrase. However, other possible (or additional) mechanisms to generate plasmid negative supercoiling in *A. profundus* cannot be ruled out at present. Treatment of *A. profundus* cultures with the gyrase inhibitor novobiocin, even at high doses (100 μg/ml), did not produce a significant effect on pGS5 supercoiling (σ varied from −0.033 ± 0.005 to −0.026 ± 0.005) (Fig. 2). Since novobiocin did not affect growth (data not shown), this result cannot be actually interpreted in terms of gyrase inhibition, and experiments with alternative inhibitors and/or a biochemical analysis of a putative gyrase should be carried out to elucidate this point.

An alternative or additional possibility to explain the negative supercoiling observed is the participation of DNA-binding proteins. Indeed, growth at low temperatures or cold shock induces plasmid negative supercoiling in *Sulfolobus* spp., which are devoid of gyrase, and which are otherwise relaxed to positively supercoiled (10). This is possibly due, at least in part, to the binding of small DNA-binding proteins of the Sso7 family (11). Unlike bacteria, including *T. maritima* (14), *Archaeoglobus* spp. possess histones (9). Archaeal nucleosomes are known to constrain negative DNA supercoils at physiological salt levels and temperature (13). However, other hyperthermophilic euryarchaeota, which are endowed with histones but devoid of gyrase, have relaxed or positively supercoiled plasmids (2, 10). *Archaeoglobus* spp. are the only known organisms where reverse gyrase, gyrase, and histones appear to coexist naturally, providing a very particular machinery to modulate DNA. Plasmid-bearing *Archaeoglobus* strains could thus be very helpful to analyze the interplay among these elements in vivo.

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