Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 10606–10611, September 1997
Biochemistry

Both DNA gyrase and reverse gyrase are present in the hyperthermophilic bacterium *Thermotoga maritima*

OLIVIER GUIPAUD*†, EVELYNE MARGUET*, KENNETH M. NOLL‡, CLAIRE BOUTHIER DE LA TOUR§, AND PATRICK FORTERRE*

*Laboratoire de Biologie Moléculaire du Gène chez les Extrémophiles, and §Laboratoire d'Enzymologie des Acides Nucléiques, Institut de Génétique et Microbiologie, Université Paris-Sud, 91405 Orsay Cedex, France; and ‡Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269

Communicated by James C. Wang, Harvard University, Cambridge, MA, July 29, 1997 (received for review May 15, 1997)

ABSTRACT **Like all hyperthermophiles yet tested, the bacterium *Thermotoga maritima* contains a reverse gyrase. Here we show that it contains also a DNA gyrase. The genes *top2*A and *top2B* encoding the two subunits of a DNA gyrase-like enzyme have been cloned and sequenced. The Top2A (type II DNA topoisomerase A protein) is more similar to GyrA (DNA gyrase A protein) than to ParC [topoisomerase IV (Topo IV) C protein]. The difference is especially striking at the C-terminal domain, which differentiates DNA gyrases from Topo IV. DNA gyrase activity was detected in *T. maritima* and purified to homogeneity using a novobiocin-Sepharose column. This hyperhermophilic DNA gyrase has an optimal activity around 82–86°C. In contrast to plasmids from hyperthermophilic archaea, which are from relaxed to positively supercoiled, we found that the plasmid pRQ7 from *Thermotoga* sp. RQ7 is negatively supercoiled. pRQ7 became positively supercoiled after addition of novobiocin to cell cultures, indicating that its negative supercoiling is due to the DNA gyrase of the host strain. The findings concerning DNA gyrase and negative supercoiling in Thermotogales put into question the role of reverse gyrase in hyperthermophiles.**

Prokaryotes possess atypical DNA topoisomerases, DNA gyrase, and reverse gyrase, which can produce negative or positive DNA supercoiling, respectively (1). Up to now, reverse gyrase has been found in all organisms with an optimal growth temperature of 80°C or above (hyperthermophiles) (2, 3), whereas DNA gyrase has been found only in mesophilic organisms. Reverse gyrase is present in both Archaea and Bacteria (the two prokaryotic domains), whereas DNA gyrase has only been isolated from bacteria, although putative DNA gyrase genes are present in halophilic archaea (4). The existence of reverse gyrase in hyperthermophilic archaea has been correlated with a unique topological state of their genetic elements (plasmid and viral DNA) which are from relaxed to positively supercoiled, whereas plasmids in mesophilic bacteria and archaea are negatively supercoiled (5–8).

Reverse gyrase and DNA gyrase appear to be specialized DNA topoisomerases. Reverse gyrase is a unique combination of a classical type I DNA topoisomerase (Topo I) and a protein module with helicase signatures (9–12). The putative ATP-dependent unwinding activity of this module probably induces the formation of a compensatory positive superturn, which is then stabilized by formation of a new topological link introduced by the topoisomerase moiety (9, 12). By contrast, DNA gyrase belongs to the classical family of Topo II (Topo IIA, see below), but it has the unique ability to wrap a DNA segment into a positive toroidal superturn (13). This enzyme is composed of two subunits, GyrA and GyrB (DNA gyrase A and B proteins), which are homologous to the C and E proteins of Topo IV (ParC and ParE), and to the C- and N-terminal halves of the eukaryotic Topo II, respectively (14). The wrapping of DNA around DNA gyrase is dependent on the C-terminal domain of the GyrA subunit (15). The positive superturn thus created introduces a compensatory negative superturn in the portion of the DNA molecule not bound to the enzyme. This positive superturn is then converted into a second negative superturn by the strand passage activity of the enzyme (16). Removal of the C-terminal domain of GyrA transforms *Escherichia coli* DNA gyrase into a classical Topo II without supercoiling activity (17).

It has been postulated that the presence of reverse gyrase is an adaptation to life at high temperature (18). Because positive supercoiling corresponds to an increase in the linking number (Lk) of topologically closed DNA molecules, it is indeed conceivable that such an Lk increase counteracts the effect of temperature elevation on the DNA molecule, maintaining either the double helical pitch and/or a more subtle parameter (DNA flexibility, unwinding potential) at an optimal value (3). By analogy, the presence of a DNA gyrase in a given organism, by decreasing the Lk, could be an adaptation to low temperatures (3). In this hypothesis, DNA gyrase and reverse gyrase might have originated in prokaryotes in the course of their adaptation to a wide range of temperatures. In this model, hyperthermophiles are predicted to have no DNA gyrase activity. In agreement with this view, the complete genome sequence of the hyperthermophilic archaeon *Methanococcus jannashii* does not contain DNA gyrase genes (19). Instead, hyperthermophilic archaea contain novel Topo II (called Topo VI) which have no DNA gyrase activity and are the prototypes of a new Topo II family (Topo IIB) with no similarity with Topo II from the classical family (Topo IIA) (20).

To determine the status of Topo II in hyperthermophilic bacteria, we have initiated the study of these enzymes in the bacterium *Thermotoga maritima*. We have previously reported the cloning and sequencing in this organism of *top2B*, a homolog of the bacterial *gyrB* and *parE* genes (21). From phylogenetic analysis of this gene, it was impossible to determine whether it encodes a DNA gyrase or a Topo IV subunit (21). Here, we report the cloning and sequencing of the *top2A* gene from *T. maritima* and the purification of a DNA gyrase from this organism. In addition, we found that a plasmid from a related Thermotogale species is negatively supercoiled. These findings challenge the correlation previously noticed

Abbreviations: GyrA, GyrB, DNA gyrase A and B proteins, respectively; ParC, ParE, DNA topoisomerase IV C and E proteins, respectively; Topo I, type I DNA topoisomerase; Topo II, type II DNA topoisomerase; Topo IIA, Topo IIB, type II DNA topoisomerase from the A and B families, respectively; Topo IV; DNA topoisomerase IV; Topo VI, DNA topoisomerase VI.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U76417).

†To whom reprint requests should be addressed. e-mail: guipaud@igmors.u-psud.fr.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

between the DNA topological state and the optimal growth temperature of the same organism (3). Moreover, these findings suggest a correlation between the existence of DNA gyrase and negative supercoiling (in the organisms that carry one).

MATERIALS AND METHODS

Bacterial Strains. Frozen cells of *T. maritima* (strain MSB8) were generously provided by R. Huber (Regensburg, Germany). *Thermotoga* sp. strain RQ7, which contains the cryptic miniplasmid pRQ7, has been described (22). *E. coli* DH5α was used as a recipient for transformation. *E. coli* C600 was used to monitor DNA gyrase purification on a novobiocin-Sepharose column.

Cell Culture. *Thermotoga* sp. strain RQ7 was cultured anaerobically at 80°C in 50 ml VSM medium (23) supplemented by 0.5% glucose and with cysteine omitted. The MIC (minimal inhibitory concentration) of novobiocin (Sigma) was obtained by cultivation of *Thermotoga* sp. RQ7 for 16 h at 80°C in the presence of various novobiocin concentrations.

Isolation of Genomic and Plasmid DNAs. Chromosomal DNA of *T. maritima* (strain MSB8) was isolated from the crude extract with the phenol procedure as described by Charbonnier *et al.* (6), except that 1 mg/ml lysozyme was added for 1 h at 37°C for the cell lysis step. Plasmid DNA from *Thermotoga* sp. strain RQ7 was isolated by the classical alkaline lysis miniprep method excepted that the time of the NaOH lysis step was reduced from 5 min to 30 s to prevent genomic DNA degradation.

Isolation of a *top2A* DNA Fragment from *T. maritima* Using PCR. Genomic DNA of *T. maritima* (strain MSB8) was amplified using degenerate oligonucleotides as primers. The sense primer 5′-ATYAAYAARCCNGTNGARGAYGA-3′ corresponded to INKPVEDE of the N-terminal sequence reported by Palm *et al.* (24), whereas the antisense primer 5′-GTNGMCATNCCNACNGCRATNCC-3′ corresponded to the well-conserved block GIAVGM(A/S)T in known GyrA and ParC proteins. The PCR was carried out in the presence of 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 2 nmol of each primer, 300 ng of *T. maritima* genomic DNA, and 2.5 units of *Taq* DNA polymerase in a volume of 100 μl. The program used was as follows: 95°C for 30 s, 55°C for 30 s, 72°C for 1 min (30 cycles) followed by a final extension step for 5 min at 72°C. Analysis of the PCR products on a 1.2% agarose gel gave two major bands of about 400 and 500 bp, which was in agreement with the expected size of the *top2A* fragment. These fragments were subcloned into pUC18 (SureClone Ligation Kit; Pharmacia) and sequenced using the dideoxy chain termination method (Sequenase Version 2.0 DNA Sequencing Kit, United States Biochemical). Analysis of the DNA sequences revealed that only the 500-bp fragment was a putative GyrA/ParC-like gene.

Cloning and Sequencing of Genomic DNA Fragments Containing the *T. maritima top2A* Gene. The 500-bp cloned PCR product labeled with [^{32}P]dCTP was found to hybridize specifically with a *Hin*dIII fragment of about 3.5 kb. The 3.5-kb genomic *Hin*dIII-digested DNA fragments were subsequently cloned into the *Hin*dIII site of pUC18 and introduced into *E. coli* DH5α. Out of 250 transformed colonies, 2 clones hybridized with the labeled probe. The restriction map of these recombinant clones were checked, and one of the *Hin*dIII inserts (3.5-kb fragment) was then sequenced (Euro Services Gènes Séquences, France). Sequence analysis showed that the 3′ part of the *top2A* gene was missing. To isolate the downstream region of the *top2A* gene, a probe corresponding to the *Spl*I/*Hin*dIII region located on the 3′ end of the 3.5-kb *Hin*dIII fragment was found to hybridize with a *T. maritima Acc*I/*Sac*I fragment of about 3.5 kb. We checked that this *Acc*I/*Sac*I 3.5-kb fragment did not hybridize with any fragment present in the middle part of the 3.5-kb *Hin*dIII fragment. This genomic region was then cloned into pUC18 digested with *Acc*I/*Sac*I. One positive clone was found out of about 2,000 colonies screened with the *Spl*I/*Hin*dIII probe. This 3.5-kb *Acc*I/*Sac*I insert was then sequenced (Euro Services Gènes Séquences).

DNA Gyrase Purification. The purification of DNA gyrase from either *T. maritima* (strain MSB8) or *E. coli* (strain C600) was carried out as described by Staudenbauer and Orr (25) with the following modifications: frozen cells (10 g) were sonicated in ice-cold 50 mM Tris·HCl (pH 7.5) containing 2 mM DTT and 1 mM phenylmethylsulfonyl fluoride. KCl and magnesium acetate concentrations of the crude extract were adjusted to 0.66 M and 5 mM, respectively, and the extract was then centrifuged for 1.5 h at 30,000 rpm in a Beckman 70 Ti rotor. Solid ammonium sulfate was added to the supernatant to reach 35% saturation (0.194 mg/ml). After stirring for 1 h at 4°C, the precipitate was remove by 20 min of centrifugation at 10,000 rpm in a Beckman JA20 rotor. The ammonium sulfate concentration in the supernatant was then adjusted to 55% by adding 0.118 g of the salt per ml. After 1 h of stirring at 4°C, the precipitate was collected by centrifugation and resuspended in 10 ml of 25 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 10% ethylene glycol, and 0.2 M KCl (buffer A). This 35–55% ammonium sulfate fraction was dialyzed overnight against buffer A and applied to a 0.7 × 2 cm novobiocin-Sepharose column (25) equilibrated with the same buffer. Proteins were first eluted with 25 mM ATP (pH 8.0) in buffer A and then with 5 M urea in buffer A. After one overnight dialysis against buffer B (50 mM potassium phosphate, pH 7.6/1 mM EDTA/1 mM DTT), the protein content of each fraction was analyzed by SDS/PAGE. The fractions were finally concentrated by dialysis against buffer B containing 50% glycerol, and aliquots were rapidly frozen in a liquid nitrogen bath and stored at −80°C. Fractions containing *T. maritima* DNA gyrase activity were identified by the supercoiling assay described below.

Supercoiling Assay. The supercoiling assay was performed in a 20-μl reaction mixture containing 500 ng of relaxed pBR322 DNA (Lucent), 35 mM Tris·HCl (pH 8.5), 1 mM EDTA, 2 mM DTT, 4 mM $MgCl_2$, 5 mM spermidine, 1 mM ATP, 0.25 mg/ml BSA, and 2 μl of each fraction eluted from the novobiocin-Sepharose column. After 3 or 10 min at 84°C, the reaction was stopped at 0°C by the addition of 1% SDS; the mixture was then incubated 15 min at 50°C with 1 mg/ml proteinase K. The samples were analyzed after electrophoresis on 1% agarose (indubiose 37 NA; Biosepra, France) gels at room temperature for 15 h at 55 V in TBE buffer (100 mM Tris-borate/2 mM EDTA). The gels were stained with ethidium bromide and were examined under UV illumination.

Topological Analysis of pRQ7. The plasmid pRQ7 was isolated from cells in late exponential phase (optical density, 0.36 at 600 nm). Cell growth was arrested by putting the cells in an Erlenmeyer flask maintained in a bath of alcohol and dry ice (23). For topological analysis in the presence of novobiocin, the antibiotic was added at a concentration of 5 μg/ml in late exponential phase (optical density, 0.36 at 600 nm) and the cells were cultivated for 30 min in the presence of the drug before isolation of pRQ7. Topology of the plasmids was analyzed by horizontal slab 2% indubiose gel two-dimensional electrophoresis, in the presence of 1 and 2 μg/ml chloroquine in the first and second dimensions, respectively. Electrophoresis was performed at room temperature in 0.5× TBE buffer (50 mM Tris-borate/1 mM EDTA) for 16 h at 40 V in the first and second dimensions. Gels were stained with ethidium bromide and examined under UV illumination.

Computer Analysis. Percentages of amino acid identity were obtained from the multiple alignment of GyrA and ParC sequences using the CLUSTAL W program (Genetics Computer Group package, version 1.6).

RESULTS

Cloning of *T. maritima top2A* Gene. We have previously cloned by PCR and sequenced a *gyrB/parE*-like gene (*top2B*) from *T. maritima* (21). This gene encodes a polypeptide that was first detected by its copurification with *T. maritima* RNA polymerase (24). In many bacteria and in the archaea of the genus *Haloferax*, the gene encoding GyrA is located downstream of GyrB. We analyzed sequences upstream and downstream of the *T. maritima top2B* gene, but no *gyrA* sequence could be detected (21). To search for the *T. maritima top2A* gene (*gyrA/parC*-like), we thus used two degenerate primers for PCR experiments (see *Materials and Methods*). The sense primer was based on the peptide sequence determined by Palm *et al.* (24) for the second polypeptide which comigrated with *T. maritima* RNA polymerase and exhibited similarities with the N-terminal region of GyrA/ParC. The antisense primer was deduced from a highly conserved region common to all bacterial GyrA and ParC. Two major fragments of the expected size were amplified, cloned, and sequenced. One of them (513 bp) was revealed as a putative *gyrA/parC* gene. This fragment was used to isolate the full-length *top2A* gene from *T. maritima* genomic DNA (see *Materials and Methods*). The *top2A* gene encodes a protein of 804 amino acids that exhibited 39–45% identity with GyrA subunits, the first 18 amino acids corresponding to the microsequence reported by Palm *et al.* (24). This result shows that the *top2A* gene indeed encodes the polypeptide that copurified with *T. maritima* RNA polymerase. Interestingly, we detected an ORF downstream of *T. maritima top2A* that encodes a protein of 197 amino acids and exhibits 25–45% identity with bacterial LexA proteins, whereas an ORF upstream of *top2A* might correspond to a gene encoding a methionyl-tRNA synthethase (unpublished data). Fig. 1 summarizes the arrangement of the *T. maritima* genome around the *top2A* and *top2B* genes.

The Top2A Protein Is More Related to GyrA Than to ParC. As described in Fig. 2, sequence comparisons of GyrA and ParC show that all these proteins have significant similarities because identities for each pair are always greater than the usually admitted significance score of 25%. However, when this analysis was restricted to the C-terminal regions (Fig. 2), a large difference was observed between DNA gyrases and Topo IV. The C-terminal sequences of GyrA appear to be well conserved because identities varied to a large extent but still remained higher than 23%. On the contrary, the identities between Topo IV, which also varied to a large extent, could go down as low as 7%. Thus, the C-terminal sequences of Topo IV appear to be very divergent. This apparent lack of sequence conservation strongly suggests that the C-terminal part of ParC is not important for the protein's function; this is in good agreement with biochemical data. Indeed, the removal of the

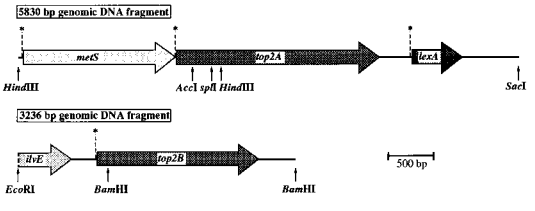

FIG. 1. The putative gene organization around *top2A* and *top2B*. The ORFs are indicated by arrowed boxes. The asterisks indicate the position of putative ribosome binding sites. No putative TATA box could be detected. The 3,236-bp genomic DNA fragment that contains *top2B* has been described (21). The amino acid sequences encoded by the *metS*- and *lexA*-like genes have, respectively, 27–48% and 25–45% identity with their bacterial homologs. According to multiple alignments and the location of the ribosome binding sites, the start codons of the *metS*- and *lexA*-like genes are proposed to be UUG, whereas the start codons of *top2A* and *top2B* are AUG. The stop codon of *metS* and the start codon of *top2A* are separated by only 13 bp.

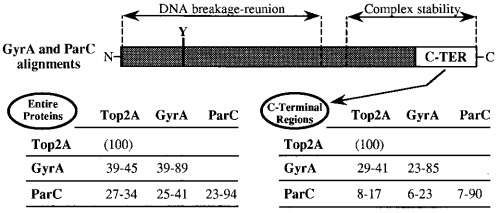

FIG. 2. Comparisons of Top2A, GyrA, and ParC primary sequences. Amino acid sequences of GyrA and ParC proteins were obtained from both SwissProt and GenBank databases. Amino acid identities (expressed in percent) were obtained by the CLUSTAL W program. Y represents the position of the active tyrosine site of the different sequences (positions 118–149). The C-terminal regions (C-TER) were chosen from positions 707–840 among these sequences. GyrA sequences are as follows: Halophile: *Haloferax* sp. phenon K; Cyanobacterium: *Synechocystis* sp. PCC6803; Gram$^+$ bacteria: *Mycobacterium leprae*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Streptomyces coelicolor*, *Bacillus subtilis*, *Staphylococcus aureus*, *Clostridium acetobutylicum*, *Mycoplasma genitalium*; Proteobacteria: *Rickettsia prowazekii*, *Neisseria gonorrhoeae*, *Helicobacter pylori*, *Campylobacter fetus*, *Campylobacter jejuni*, *Escherichia coli*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Erwinia carotovora*, *Klebsiella pneumoniae*, *Aeromonas salmonicida*. ParC sequences are as follows: Cyanobacterium: *Synechocystis* sp. PCC6803; Gram$^+$ bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Mycoplasma genitalium*; Proteobacteria: *Neisseria gonorrhoeae*, *Escherichia coli*, *Salmonella typhimurium*, *Haemophilus influenzae*.

C-terminal domain of GyrA transforms *E. coli* DNA gyrase into a classical Topo II (17). On the contrary, the C-terminal domain of DNA gyrase, which wraps the DNA into a positive superturn, is critical for gyrase activity (15–17). The comparison of Topo IV with DNA gyrases or with *T. maritima* Top2A in this C-terminal region led to no significant scores, all less than 23%. On the contrary, the percentage identities between *T. maritima* Top2A and DNA gyrases was always larger than 29% (Fig. 2). Thus, analysis of *T. maritima* Top2A strongly suggests that this protein is a DNA gyrase subunit.

Purification of *T. maritima* DNA Gyrase. To confirm that *T. maritima* indeed contains a DNA gyrase, we have purified this enzyme by affinity chromatography using a novobiocin-Sepharose column (25). To check our column, we first purified *E. coli* DNA gyrase (Fig. 3*A*). An *E. coli* cell extract was loaded onto the novobiocin-Sepharose column and bound proteins were eluted first by 20 mM ATP and then by 5 M urea. As shown on SDS gel (Fig. 3*A*), the GyrA subunit (105 kDa) was eluted prior to GyrB (92 kDa), which was directly bound to the column by its affinity for novobiocin. The *E. coli* DNA gyrase activity was observed in the first fractions of the urea wash step when both GyrA and GyrB subunits were eluted together (data not shown).

We used the same protocol to purify a *T. maritima* Topo II. A topoisomerase activity was detected in the fractions eluted by 5 M urea (Fig. 3*B*). This activity (tested at 84°C) was ATP-dependent and strongly inhibited by novobiocin (data not shown). The product of the reaction was negatively supercoiled and contained at least 15 negative superturns in the electrophoresis gel (Figs. 3*B* and Fig. 4). Considering that the change of the rotation angle of the DNA double helix with increasing temperature is $-0.010°/°C/bp$ (6, 26), we calculated that a 60°C difference of temperature (from 84°C at reaction to about 25°C at electrophoresis) would result in a change of the writhe value (Wr) by about -7 for pBR322 (4,361 bp). This finding indicates that pBR322 contained at least 8 negative superturns at the reaction temperature (84°C). Furthermore, the reaction product also contained at least 15 negative superturns at electrophoresis temperature when the reaction was performed at 40–50°C (data not shown). Our results thus clearly indicate that this *T. maritima* Topo II is a DNA gyrase.

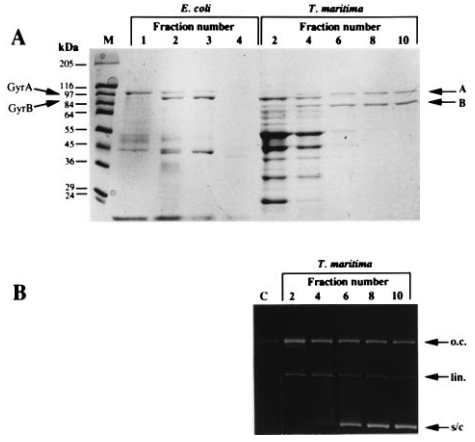

FIG. 3. Purification of the DNA gyrase from *T. maritima* using a novobiocin-Sepharose column. (*A*) SDS/PAGE of fractions containing novobiocin-binding proteins from *E. coli* and *T. maritima*. Crude extracts were dialyzed against buffer A and applied to a novobiocin-Sepharose column (see *Materials and Methods*). The column was eluted successively with buffer A containing 20 mM ATP and buffer A containing 5 M urea. Samples (10 μl) from several dialyzed fractions of the 5 M urea eluates were applied to SDS/PAGE (8%). Lane M is the wide range molecular weight marker (Sigma). GyrA and GyrB, respectively 105 and 92 kDa, are the two subunits of the DNA gyrase from *E. coli*. Markers A and B are the two subunits of the DNA gyrase from *T. maritima*. (*B*) A total of 2 μl of each fraction from the *T. maritima* 5 M urea eluates analyzed on SDS/PAGE were incubated with relaxed pBR322 at 84°C for 10 min. Samples were analyzed on a 1% indubiose gel (lane C, control; o.c., open circular; lin., linear; s/c, negatively supercoiled).

As shown on Fig. 3*A* (fractions 4, 6, 8, and 10), this DNA gyrase activity copurified with two polypeptides of 90 and 70 kDa. In the fractions that exhibit the strongest supercoiling activity (Fig. 3*B*, fractions 8 and 10), these two polypeptides represented more than 90% of the protein sample. Their sizes are in good agreement with the calculated molecular weights of the proteins encoded by *top2A* and *top2B* (90 and 72 kDa, respectively). This result strongly suggests that *top2A* and *top2B* genes encode the DNA gyrase we purified.

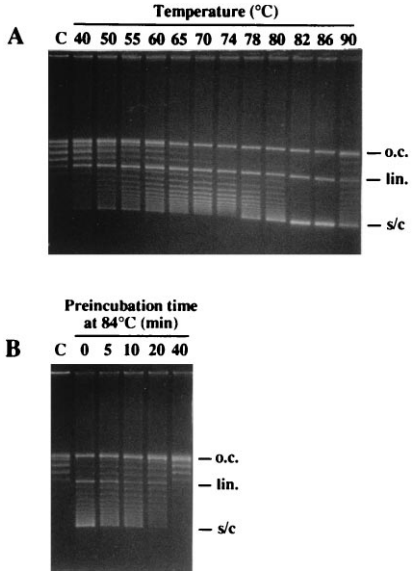

FIG. 4. Thermophily and thermostability of the *T. maritima* DNA gyrase. Relaxed pBR322 was incubated with the fraction containing active DNA gyrase for 3 min at various temperatures without preincubation (*A*) or for 3 min at 84°C after various periods of preincubation at 84°C (*B*). Samples were analyzed on a 1% indubiose gel (lane C, control; o.c., open circular; lin., linear; s/c, negatively supercoiled).

Interestingly, GyrA was not eluted at the beginning of the urea step, as in the case of the *E. coli* DNA gyrase. Indeed, the two subunits coeluted in stoichiometric amounts all through the urea wash (Fig. 3*A*), suggesting that the interactions between the two subunits are stronger than those found for *E. coli* DNA gyrase. Furthermore, some GyrA and GyrB polypeptides were still coeluted with gyrase activity after washing with 8 column volumes of 5 M urea, indicating that either this *T. maritima* DNA gyrase was more tightly bound to the novobiocin column or more resistant to unfolding by urea than *E. coli* DNA gyrase.

Thermophily and Thermostability of *T. maritima* DNA Gyrase. *T. maritima* DNA gyrase was active in the temperature range from 50°C to at least 90°C, with an optimal temperature for activity around 82–86°C (Fig. 4*A*). We observed linear DNA product in our DNA gyrase tests, even in fractions eluted after 8 column volumes, suggesting that it could be generated by uncorrectly refolded DNA gyrase molecules. We also noticed a complex pattern of change in topoisomer distribution with temperature, suggesting that temperature might affect the processivity of the enzyme, but we have not explored this phenomenon further. *T. maritima* DNA gyrase was not very stable at high temperature; its half-life was around 10 min at both the temperature corresponding to its optimal activity (84°C) (Fig. 4*B*) and at 70°C (not shown). However, this is in the range of stability observed for other enzymes from Thermotogales *in vitro*.

The Plasmid pRQ7 from Thermotoga sp. RQ7 Is Negatively Supercoiled. To get an insight into the intracellular topological state of DNA in Thermotogales, we determined the topological state of pRQ7, a small plasmid of 846 bp discovered in the strain *Thermotoga* sp. RQ7 (22). To fix the biological level of intracellular supercoiling, cells were rapidly chilled by putting the cells in an Erlenmeyer flask maintained in a bath of alcohol and dry ice (23). pRQ7 migrated as a single band in an agarose gel and was relaxed by addition of intercalating drugs (chloroquine or ethidium bromide), indicating that it was negatively supercoiled at the temperature of the electrophoresis. To determine its level of supercoiling, pRQ7 was then analyzed by two-dimensional agarose gel electrophoresis (27) in the presence of 1 and 2 μg/ml chloroquine in the first and second dimensions, respectively. In these conditions, pRQ7 was partly relaxed in the first dimension by chloroquine but still negatively supercoiled because its topoisomers formed the left side of the arch in the second dimension (Fig. 5). The superhelical density (σ) of pRQ7 was determined by comparison with pTZ18, an *E. coli* plasmid of known superhelical density (σ = -0.052; see ref. 6) run on the same two-dimensional gel (not

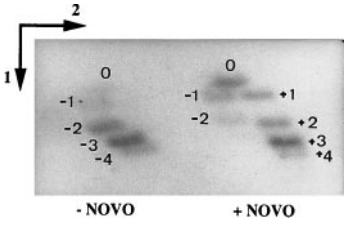

FIG. 5. Comparitive topoprofiles of pRQ7 extracted from *Thermotoga* sp. RQ7 cultivated with (+ NOVO) or without (− NOVO) novobiocin. Cells were cultivated for 16 h (late exponential phase) when 5 μg/ml novobiocin was added or not. The cells were cultivated for 30 min more before isolation of the plasmid. pRQ7 was analyzed by two-dimensional gel electrophoresis in the presence of chloroquine (see *Materials and Methods*). Arrows 1 and 2 show the direction of the run in the first and second dimensions, respectively. Topoisomers forming the left side of the arch are negatively supercoiled in the gel whereas topoisomers forming the right side of the arch are positively supercoiled in the gel (27). The numbers of superturns of each topoisomer are mentioned. The figure is a photograph of an ethidium bromide-stained gel with the image inverted to enhance contrast.

shown). The major topoisomers of pTZ18 and pRQ7 contained three and either two or three negative superturns, respectively. Considering the respective sizes of these plasmids, we calculated a superhelical density of -0.067 ± 0.005 for pRQ7 at the temperature of the electrophoresis. Taking into account the effect of temperature on the DNA winding angle (6, 26), this corresponds to an actual superhelical density at the growth temperature of *Thermotoga* sp. RQ7 (80°C) of -0.049 ± 0.005, indicating that pRQ7 is negatively supercoiled.

The Plasmid pRQ7 Became Positively Supercoiled after Novobiocin Treatment *in Vivo*. Novobiocin, a universal inhibitor of DNA gyrase, was found to inhibit the growth of *Thermotoga* sp. RQ7 with a minimal inhibitory concentration (MIC) of 5 μg/ml (data not shown). To determine if the *T. maritima* DNA gyrase was responsible for the negative supercoiling of pRQ7, we thus looked at the topology of pRQ7 after addition of 5 μg/ml novobiocin for 30 min to the cell culture. In similar experiments, plasmids from *E. coli* (28) or from halophilic archaea (29) became positively supercoiled. The interpretation is that plasmid transcription and/or replication produce positive superturns which are normally removed by DNA gyrase and accumulate when DNA gyrase is inhibited by novobiocin (30). *Thermotoga* sp. RQ7 was cultivated for 16 h (late exponential phase) and then cultivated with or without 5 μg/ml novobiocin for 30 min before plasmid isolation. To determine the level of supercoiling of pRQ7, the plasmids were then analyzed by two-dimensional agarose gel electrophoresis in the presence of 1 and 2 μg/ml chloroquine in the first and second dimensions, respectively. As seen in Fig. 5, the major topoisomer of pRQ7 isolated from cells treated with novobiocin contains three positive superturns (right side of the arch) in the presence of 1 μg/ml chloroquine, whereas three negative superturns (left side of the arch) are present in the major topoisomer of pRQ7 isolated from untreated cells. Considering the effect of chloroquine (see above) and temperature (26, 6) on DNA structure, we calculated that pRQ7 isolated from cells treated with novobiocin becomes positively supercoiled, with an estimated superhelical density at 80°C of $+0.015 \pm 0.005$. This result indicates that negative supercoiling of pRQ7 in untreated cells is produced by its DNA gyrase.

DISCUSSION

DNA gyrase was previously isolated only from mesophiles. In *E. coli*, DNA gyrase is a cold-shock protein (31), and we even speculated that gyrase was an adaptation to mesophily (3). However, we have now isolated a DNA gyrase from a hyperthermophilic organism, the bacterium *T. maritima*. Because this prokaryote can grow in a wide temperature range (55–90°C), it could be argued that its DNA gyrase may only be active at the lower end of its temperature range. This is not the case, however, because *T. maritima* DNA gyrase turned out to be highly thermophilic. It has an optimal activity around 82–86°C, which corresponds to the optimal growth temperature of *T. maritima* and is active at least up to 90°C.

T. maritima DNA gyrase is not very stable at the maximal growth temperature of its host strain, with a half-life around 10 min at 84°C, but this is common to other enzymes from Thermotogales *in vitro*. Therefore it is likely that this enzyme is stabilized *in vivo* by interaction with other cellular components. Interestingly, the two subunits of *T. maritima* DNA gyrase are more tightly bound together than *E. coli* GyrA and GyrB (see Fig. 3*A*), because they remain associated in the presence of 5 M urea. This finding suggests that additional interactions are involved in their binding. We have previously noticed that *T. maritima* Top2B contains the highest amount of charged amino acids of all GyrB/ParC known at that time (21), which fits well with the hypothesis that hyperthermophilic proteins are mainly stabilized by networks of charged amino acids at their surface (32, 33). However, we did not observe such trends with *T. maritima* Top2A. On the other hand, we have seen that this enzyme is not as stable as some proteins from hyperthermophilic Archaea, which can be resistant to temperatures of 100°C or more.

The discovery of a DNA gyrase in *T. maritima* was surprising because this bacterium, like all other hyperthermophiles, also contains a reverse gyrase (34). The gene encoding this enzyme has been recently cloned and sequenced in M. Duguet's lab (C.B.d.l.T., C. Portemer, H. Kaltoum, and M. Duguet, unpublished data), and this bacterial reverse gyrase exhibits the same dual-structure as the archaeal reverse gyrases—i.e., association of a helicase and a topoisomerase domain.

The presence of both a DNA gyrase and a reverse gyrase in the same organism raised the question of the topological state of its DNA. To get an insight into the intracellular topological state of DNA in Thermotogales, we have investigated the topological state of the plasmid pRQ7 of a related strain, *Thermotoga* sp. RQ7. We have verified that strain RQ7 contains both DNA gyrase and reverse gyrase genes by Southern blot analysis using probes prepared from the genes encoding the GyrA and GyrB subunits and the reverse gyrase of *T. maritima*. In all cases, we obtained a clear-cut signal (data not shown). We show here that pRQ7 is negatively supercoiled, resembling the topology of plasmids from mesophilic bacteria. The superhelical density of pRQ7 extrapolated to the growth temperature of the organism at the time of isolation (80°C) is similar to the typical superhelical density of *E. coli* plasmids. Granted, the true degree of supercoiling in the cell is not clear and the negative supercoiling found for the plasmid does not imply that the chromosome is also negatively supercoiled. In particular, the involvement of histone-like proteins in the torroidal superhelicity is not known for *Thermotoga* species. However, as in the case of *E. coli* and other mesophilic bacteria, a negative superhelicity of the plasmid could also indicate a negative superhelicity of the chromosome.

pRQ7 became positively supercoiled after treatment of living cells with concentrations of novobiocin that inhibit *T. maritima* DNA gyrase *in vitro*. This result indicates that DNA gyrase is most likely responsible for the negative supercoiling of this plasmid *in vivo* and predominates over reverse gyrase in *Thermotoga* sp. RQ7. We have observed that pRQ7 became relaxed at stationary phase (data not shown), indicating a possible regulation of DNA gyrase and/or reverse gyrase activity during the growth phase of this organism.

The discovery of a DNA gyrase and negative supercoiling in a hyperthermophilic bacterium was surprising because all previously described hyperthermophilic archaea have relaxed to positively supercoiled DNA and no DNA gyrase activity. This result indicates that these two features are not hallmarks of hyperthermophiles but are specific to hyperthermophilic archaea. Hyperthermophilic archaea and bacteria have thus completely different DNA topologies and patterns of topoisomerase activities. Another similarity between Thermotogales and mesophilic bacteria is the presence of a Topo I, which is closely related to the *E. coli* ω protein (35). In contrast, hyperthermophilic archaea contain a Topo I related to *E. coli* and yeast topoisomerase III (19, 36).

The intracellular topological state in Thermotogales, at least for a specific plasmid DNA, appears identical to that in mesophilic bacteria and in halophilic archaea, which both contain a DNA gyrase. Accordingly, although our results with *Thermotoga* indicate that there is no correlation between the presence of a reverse gyrase activity and the topological state of DNA, they do suggest a correlation between the presence of a DNA gyrase and the intracellular topological state—i.e., organisms with DNA gyrase have negatively supercoiled DNA. It will be interesting to look for mesophilic or moderately thermophilic organisms with no DNA gyrase activity and relaxed DNA. A putative candidate is *Methanobacterium ther-*

moautotrophicum, which has a relaxed plasmid (pME2001) and no reverse gyrase activity (7).

We have previously proposed a model in which DNA gyrase and reverse gyrase appeared in the course of prokaryotic evolution to counteract the effect of temperature on DNA structure (3). However, the presence of DNA gyrase and negative supercoiling seems to be of no disadvantage for a hyperthermophile such as *Thermotoga*. This observation presents a challenge to the previous model that reverse gyrase functions to oversupercoil DNA in response to high temperatures. Nevertheless, although *Thermotoga* seems to have negatively supercoiled DNA, at least for a specific plasmid DNA, this does not rule out the hypothesis that reverse gyrase functions to push DNA supercoiling in the positive direction in the case of higher temperatures. In this respect it will be interesting to learn what happens to levels of both reverse gyrase and DNA gyrase as a function of temperature as the appropriate reagents become available (e.g., antibodies).

What can be the role of reverse gyrase in hyperthermophiles if these organisms can tolerate negative supercoiling? Is it really related to hyperthermophily? A positive answer is suggested by the presence of this enzyme in all hyperthermophiles (including those with a DNA gyrase) and its absence in all mesophiles tested up to now. However, it might be worth continuing to screen for reverse gyrase in archaea to be sure that it is not present for example in the mesophilic Crenarchaeota recently discovered in various cold environments or in mesophilic relatives of the Thermotogales. It is possible that reverse gyrase does not act on global supercoiling but counteracts specific processes that could be destabilizing at high temperature, such as DNA unwinding after the passage of transcription forks or during the branch migration of recombination junctions (37). Reverse gyrase could also be used as a "reformatase" to eliminate a variety of abnormal DNA structures that could be favored by high temperature (such as cruciform structures) and that can be destabilized by positive supercoiling (37, 38).

The role of reverse gyrase *in vivo* is presently difficult to assess because of the absence of genetic tools for hyperthermophiles. Hyperthermophilic bacteria are interesting model systems for further studies in that direction since the development of genetic systems in these organisms could be easier than in archaea. Finally, the presence of both DNA gyrase and reverse gyrase in Thermotogales makes these microorganisms an attractive model system to study the unique regulatory interaction of these two enzymes in the same organism.

Note Added in Proof. Analysis of recently released sequence data indicates that the complete genome of the hyperthermophilic archaeon *Archaeoglobus fulgidus* also contains genes encoding both a reverse gyrase and a type II DNA topoisomerase specifically related to DNA gyrase.

We thank Robert Huber (Regensburg, Germany) for the gift of *T. maritima* cells, and also Cedric Petit and Isabelle Guillemin. We are grateful to Agnès Bergerat and Bernard Labedan for preparation of this manuscript. This work was supported by grants from la Ligue Nationale contre le Cancer, l'Association pour la Recherche sur le Cancer, the European Community Program Cell Factory (BIO 4-CT 96-0488), and the National Science Foundation (MCB 9418197).

1. Wang, J. C. (1996) *Annu. Rev. Biochem.* **65,** 635–692.
2. Duguet, M. (1995) in *Nucleic Acids and Molecular Biology*, eds. Eckstein, F. & Lilley, D. M. J. (Springer, Berlin), Vol. 9, pp. 84–114.
3. Forterre, P., Bergerat, A. & López-García, P. (1996) *FEMS Microbiol. Rev.* **18,** 237–248.
4. Holmes, M. L. & Dyallsmith, M. L. (1991) *J. Bacteriol.* **173,** 642–648.
5. Nadal, M., Mirambeau, G., Forterre, P., Reiter, W. D. & Duguet, M. (1986) *Nature (London)* **321,** 256–258.
6. Charbonnier, F., Erauso, G., Barbeyron, T., Prieur, D., Forterre, P. (1992) *J. Bacteriol.* **174,** 6103–6108.
7. Charbonnier, F. & Forterre, P. (1994) *J. Bacteriol.* **176,** 1251–1259.
8. López-García, P. & Forterre, P. (1997) *Mol. Microbiol.* **23,** 1267–1279.
9. Confalonieri, F., Elie, C., Nadal, M., Bouthier de la Tour, C., Forterre, P. & Duguet, M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4753–4757.
10. Krah, R., Kozyavkin, S. A., Slesarev, A. I. & Gellert, M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 106–110.
11. Borges, K. M., Bergerat, A., Bogert, A. M., DiRuggiero, J., Forterre, P. & Robb, F. T. (1997) *J. Bacteriol.* **179,** 1721–1726.
12. Jaxel, C., Bouthier de la Tour, C., Duguet, M. & Nadal, M. (1996) *Nucleic Acids Res.* **24,** 4668–4675.
13. Liu, L. F. & Wang J. C. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2098–2102.
14. Caron, P. R. & Wang, J. C. (1994) in *DNA Topoisomerases, Topoisomerase-Targeting Drugs*, ed. Liu, L. F. (Academic, New York), Vol. 29A, pp. 271–297.
15. Reece, R. J. & Maxwell, A. (1991) *Nucleic Acids Res.* **19,** 1399–1405.
16. Cozzarelli, N. R. (1980) *Science* **207,** 953–960.
17. Kampranis, S. C. & Maxwell, A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 14416–14421.
18. Kikuchi, A. & Asai, K. (1984) *Nature (London)* **309,** 677–681.
19. Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., *et al*. (1996) *Science* **273,** 1058–1073.
20. Bergerat, A., de Massy, B., Gadelle, D., Varoutas, P. C., Nicolas, A. & Forterre, P. (1997) *Nature (London)* **386,** 414–417.
21. Guipaud, O., Labedan, B. & Forterre, P. (1996) *Gene* **174,** 121–128.
22. Harriott, O. T., Huber, R., Stetter, K. O., Betts, P. W. & Noll, K. M. (1994) *J. Bacteriol.* **176,** 2759–2762.
23. Marguet, E., Zivanovic, Y. & Forterre, P. (1996) *FEMS Microbiol. Lett.* **142,** 31–36.
24. Palm, P., Schleper, C., Arnoldammer, I., Holz, I., Meier, T., Lottspeich, F. & Zillig, W. (1993) *Nucleic Acids Res.* **21,** 4904–4908.
25. Staudenbauer, W. L. & Orr, E. (1981) *Nucleic Acids Res.* **9,** 3589–3603.
26. Depew, R. E. & Wang, J. C. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 4275–4279.
27. Wang J. C., Peck L. J. & Becherer K. (1983) *Cold Spring Harbor Symp. Quant. Biol.* **47,** 85–91.
28. Lockson, D. & Morris, D. R. (1983) *Nucleic Acids Res.* **11,** 2999–3017.
29. Sioud, M., Baldacci, G., De Recondo, A. M. & Forterre, P. (1988) *Nucleic Acids Res.* **16,** 1379–1392.
30. Liu, L. F. & Wang J. C. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7024–7027.
31. Jones, P. G., Krah, R., Tafuri, S. R. & Wolffe, A. P. (1992) *J. Bacteriol.* **174,** 5798–5802.
32. Sterner, R., Dahm, A., Darimont, B., Ivens, A., Liebl, W. & Kirschner, K. (1995) *EMBO J.* **14,** 4395–4402.
33. Yip, K. S. P., Stillman, T. J., Britton, K. L., Artymiuk, P. J., Baker, P. J., Sedelnikova, S. E., Engel, P. C., Pasquo, A., Chiaraluce, R., Consalvi, V., Scandurra, R. & Rice, D. W. (1995) *Structure* **3,** 1147–1158.
34. Bouthier de la Tour, C., Portemer, C., Huber, R., Forterre, P. & Duguet, M. (1991) *J. Bacteriol.* **173,** 3921–3923.
35. Bouthier de la Tour, C., Kaltoum, H., Portemer, C., Confalonieri, F., Huber, R. & Duguet, M. (1995) *Biochim. Biophys. Acta* **1264,** 279–283.
36. Slesarev, A. I., Zaitzev, D. A., Kopylov, V. M., Stetter, K. O. & Kozyavkin, S. A. (1991) *J. Biol. Chem.* **266,** 12321–12328.
37. Kikuchi, A. (1990) in *DNA Topology and Its Biological Effects*, eds Cozzarelli, N. R. & Wang, J. C. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 285–298.
38. Duguet, M. (1997) *J. Cell Sci.* **110,** 1345–1350.