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Reverse Gyrase in Thermophilic Eubacteria

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The presence of reverse gyrase, an unusual ATP-dependent type I topoisomerase first isolated from thermophilic archaebacteria, has been detected in four strains of *Thermotogales*, an order of extremely thermophilic eubacteria. This result suggests that reverse gyrase plays a key role in high-temperature-living organisms, independently of the evolutionary kingdom to which they belong.

Reverse gyrase is a type I topoisomerase first isolated from a thermophilic archaebacterium, *Sulfolobus acidocaldarius* (7). This enzyme, fully active at 75°C, catalyzes the introduction of positive supercoils into a closed circular DNA, in an ATP-dependent process (3, 9–11). The discovery of such an unusual enzyme raised the question of its function and distribution within archaebacteria.

In a previous study, we have shown that reverse gyrase activity was largely distributed among thermophilic archaebacteria, suggesting that the enzyme is needed for life at high temperature (1). However, the enzyme seemed restricted to archaebacteria, since a preliminary result with *Thermotoga maritima*, an extremely thermophilic eubacterium, was negative (1). Indeed, reverse gyrase was masked in this strain by a prominent ATP-independent relaxation activity, together with an important nucleolytic activity.

In the present paper, we provide evidence that the enzyme is present in thermophilic eubacteria. We found a reverse gyrase-like activity in four different strains of the order *Thermotogales*, which includes the most extremely thermophilic eubacteria known.

The four isolates used in this study were *Thermotoga maritima* DSM 3109 (4) and *Thermotoga thermarum* LA3 (13) (growth temperature: 80°C), *Fervidobacterium islandicum* H21 (6), and *Thermosipho africanus* Ob7 (5) (growth temperature, 75°C). Preparation of the partially purified fractions from these strains was as previously described (1). Briefly, the cells were suspended at 200 mg/ml in buffer A (50 mM Na_2HPO_4-NaH_2PO_4, pH 7.0, 1 mM dithiothreitol, 1 mM EDTA) containing 1.5 M NaCl, 1.0 M $(NH_4)_2SO_4$, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid] 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium bisulfite, 1 μg of leupeptin per ml, and 1 μg of pepstatin A per ml. The cells were then lysed by ultrasonication, and their content was collected by centrifugation (12,000 × *g*, 15 min). Partial purification was achieved by phenyl Sepharose chromatography; after binding at high salt, the proteins were step eluted with increasing ethylene glycol concentrations. In all cases, topoisomerase activity was mainly recovered in the 60% ethylene glycol fraction.

In the case of *F. islandicum*, further purification was performed by chromatography on phosphocellulose. Proteins from the ethylene glycol fraction were dialyzed against buffer A containing 15% ethylene glycol and 0.1 M NaCl (buffer B), before being loaded onto the column (Whatman P 11) equilibrated with this buffer. Then, the column was washed with 10 volumes of buffer B, and the bound proteins were finally step eluted with 1.0 M NaCl in buffer B. Protein concentrations were measured as described by Schaffner and Weissmann (12).

Topoisomerase assays were performed as previously described (1), except that the DNA substrate was pTZ 18 (8): the standard reaction mixture (20 μl) contained 50 mM Tris HCl, pH 8.0, 0.5 mM dithiothreitol, 0.5 mM EDTA, 10 mM $MgCl_2$, 1.0 mM ATP, 30 or 150 mM NaCl, 30 μg of bovine serum albumin per ml, 6% ethylene glycol, and 600 ng of negatively supercoiled pTZ 18 DNA. After addition of 135 ng of partially purified fraction, the mixture was incubated at 75°C for 30 min. The products of incubation were analyzed by two-dimensional gel electrophoresis, following the procedure previously described (3), in order to distinguish between negatively and positively supercoiled topoisomers.

The results are presented in Fig. 1. When incubation was performed at low ionic strength (30 mM NaCl), a noticeable reverse gyrase activity appeared in the case of *F. islandicum* and *Thermotoga thermarum* (panels B and C). Production of positive supercoils was clearly ATP dependent. However, an important ATP-independent relaxation activity was also present (columns −). In contrast (panel D), positive supercoiling appeared weak in the case of *Thermosipho africanus* and undetectable in the case of *Thermotoga maritima* when 135 ng of the phenyl Sepharose fraction was used (not shown). For the latter, detection of positive supercoiling was likely prevented by the presence of a nuclease activity and probably a low reverse gyrase content in this strain. Nevertheless, when a twofold amount of *Thermotoga maritima* fraction was used, a faint, but significant, positive supercoiling was detected (panel E), despite the presence of nuclease, indicated by appearance of linear DNA.

The situation was clearly difference when incubations were performed at higher concentrations of salt (150 mM NaCl, panels F to I): in this case, *F. islandicum* and *Thermotoga thermarum* (panels F and G) exhibited a reduced positive supercoiling activity, together with a reduced ATP-independent relaxation activity. For *Thermosipho africanus* and *Thermotoga maritima*, positive supercoiling was completely abolished at 150 mM NaCl, while the ATP-independent relaxation seemed enhanced (panels H and I).

Finally, we asked whether the ATP-independent relaxing

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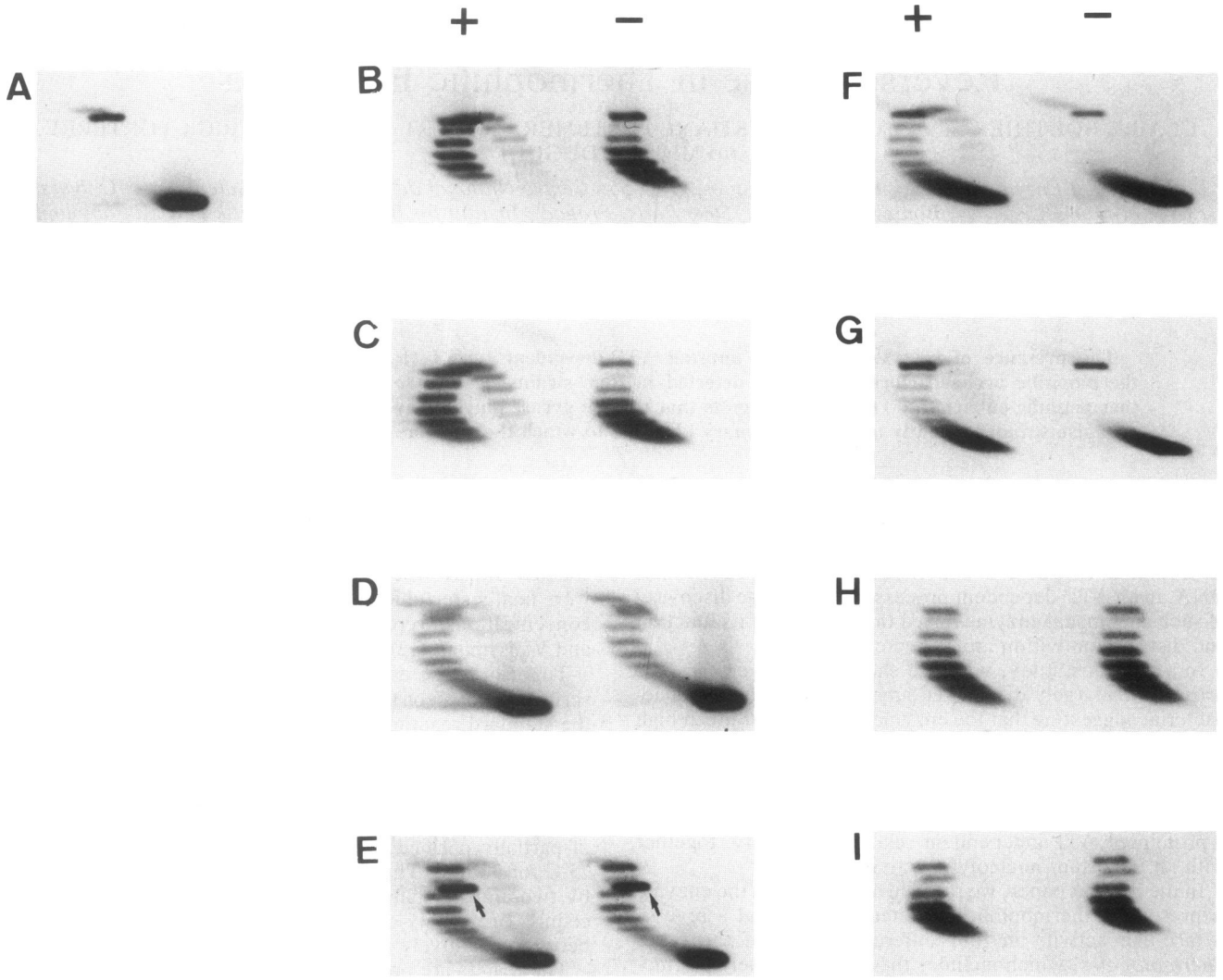

FIG. 1. Bidimensional gel analysis of the topological conversions of pTZ 18 DNA after incubation with semi-purified fractions from various strains of the order *Thermotogales*. The assays were performed in the presence (+) or absence (−) of ATP. (A) pTZ 18 DNA control (600 ng). Three bands are visible: negatively supercoiled DNA (bottom band); open circular DNA (top band); and negatively supercoiled dimer (extreme left band). (B to I) pTZ 18 (600 ng) was incubated at 30 mM (panels B to E) or 150 mM (panels F to I) NaCl with 135 ng of fractions from *F. islandicum* (B and F), *Thermotoga thermarum* (C and G), *Thermosipho africanus* (D and H), and 270 ng of fraction from *Thermotoga maritima* (E and I). On each panel, the left branch of the arch corresponds to negatively supercoiled topoisomers and the right branch corresponds to positively supercoiled topoisomers. Linear DNA (form III) is indicated by an arrow.

activity was an intrinsic property of reverse gyrase or was carried by another topoisomerase. The latter hypothesis is consistent with our findings: indeed, when the 60% ethylene glycol fraction of *F. islandicum* was further purified on phosphocellulose, the activity of the fraction eluted at 1.0 M NaCl was almost completely ATP dependent (Fig. 2A), while the ATP-independent relaxing activity was not retained on the column at 0.1 M NaCl (Fig. 2B).

The results reported in this paper show that, contrary to our previous observation (1) and those of Collin et al. (2), a reverse gyrase-like activity takes place in members of the order *Thermotogales*. In the case of *Thermotoga maritima*, there were several reasons that this activity might go undetected. First, the presence of nuclease led us to perform incubations at 150 mM NaCl, where reverse gyrase was less active. Second, reverse gyrase activity was low in this strain. Third, an ATP-independent topoisomerase activity was present in addition to reverse gyrase and was the major topoisomerase activity in *Thermotoga maritima*. Thus, one must be careful in analyzing topoisomerase activities in a bacterial strain, since the results are largely dependent on the conditions used.

In the case of *F. islandicum*, ATP-dependent reverse gyrase could be separated from ATP-independent relaxation activity on a phosphocellulose column, suggesting that at least two distinct topoisomerases exist in these species, both able to relax negatively supercoiled DNA. Partially purified reverse gyrase was almost completely ATP dependent. In addition, a very low amount (4 ng) of the phosphocellulose fraction was sufficient to produce efficient positive supercoiling, suggesting that the reaction was catalytic. In order to further characterize the properties of this enzyme and to

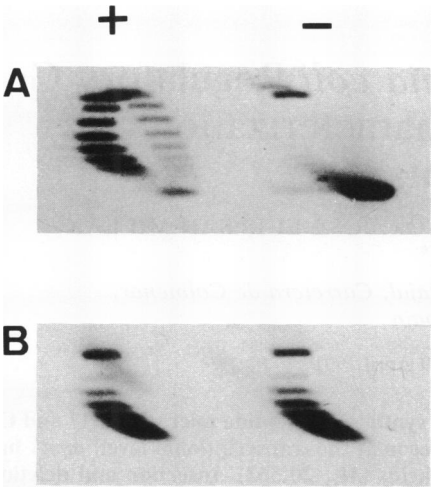

FIG. 2. Bidimensional analysis of pTZ 18 after incubation with the fraction of *F. islandicum* eluted from phosphocellulose. The assay was performed as described in the text: 600 ng of pTZ 18 was incubated at 30 mM NaCl in the presence (+) or absence (−) of ATP. (A) pTZ 18 after incubation with 4 ng of the 1.0 M NaCl fraction from phosphocellulose. (B) pTZ 18 after incubation with 4 ng of the fraction not retained on phosphocellulose at 0.1 M NaCl.

compare these with the properties of the archaebacterial reverse gyrase, it is necessary to purify this protein to homogeneity.

The existence of reverse gyrase in thermophilic eubacteria supports the idea that this enzyme may play an important function in organisms living at high temperature, independently of the evolutionary domain they belong to. This hypothesis is consistent with the apparent lack of reverse gyrase activity in a number of mesophilic strains previously tested (1). The in vivo function of this enzyme is not clearly demonstrated: although the DNA of the viruslike particle SSV1 extracted from *Sulfolobus* sp. strain B12-infected cells appeared positively supercoiled (10), there is no direct evidence for the involvement of this type of supercoiling in the stability of DNA at high temperature.

The finding of reverse gyrase in both eubacteria and archaebacteria also suggests that it may be an ancestral protein which appeared very early during evolution, before the divergence between eubacteria and archaebacteria (14).

Finally, since topoisomerase properties are closely linked to genome structure and evolution, the presence of reverse gyrase in archaebacterial and eubacterial domains would suggest some similarities in the structural organization of their genomes.

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