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First functional characterization of a singly expressed bacterial type II topoisomerase: The enzyme from *Mycobacterium tuberculosis*

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Abstract

Genome deciphering revealed that *Mycobacterium tuberculosis* encodes a single type II topoisomerase contrary to common bacteria harboring two type II topoisomerases (DNA gyrase and topoisomerase IV). Functions of the *M. tuberculosis* type II topoisomerase were explored after cloning and expressing the subunits encoding genes in *Escherichia coli*. *M. tuberculosis* type II topoisomerase supercoiled relaxed pBR322 with a specific activity close to that of DNA gyrases of common bacteria whereas it exhibited DNA relaxation and formation of cleavable complexes with activities significantly higher than other DNA gyrases. Intermolecular passage activity evaluated by the decatenation of kinetoplast DNA was 25-fold lower than that of the topoisomerase IV from *Streptococcus pneumoniae*, but was markedly higher than that of the *E. coli* gyrase. Overall, the type II topoisomerase of *M. tuberculosis* exhibits classical polyvalent activities of DNA gyrase for supercoiling but enhanced relaxation, cleavage, and decatenation activities.

Keywords: Topoisomerase; Decatenation; *Mycobacterium tuberculosis*; DNA gyrase

Replication of the circular bacterial chromosome requires unwinding of the topological intertwined strands of the closed DNA duplex [1–3] which must be reduced to zero to allow segregation of the two daughter chromosomes at cell division [3]. To meet this requirement, most bacteria express two ATP-dependent type II topoisomerases that are structurally and mechanistically related but have acquired distinct characteristics during evolution [1]. DNA gyrase facilitates DNA unwinding at replication forks, and topoisomerase IV has a specialized function in mediating the decatenation of interlocked daughter chromosomes [1,3]. Both enzymes act by passing one DNA segment through a transient double-strand break in a second DNA segment. The DNA segments are part of the same molecule (the chromosome) during relaxation and supercoiling required for transcription, recombination, and replication (intramolecular passage activity), and they belong to two different molecules (two daughter chromosomes) in decatenation (intermolecular passage activity).

DNA gyrase present exclusively in bacteria and mitochondria is the only type II topoisomerase that can introduce negative supercoils into DNA [1–3]. This enzyme facilitates replication fork movement by neutralizing the positive supercoils arising from helix unwinding during replication. DNA gyrase has been characterized from a number of bacterial species and is a heterotetramer composed of two GyrA subunits encoded by the *gyrA* gene, and two GyrB subunits encoded by *gyrB* [3]. The GyrA N-terminal domain contains the site for DNA breakage–reunion whereas the C-terminal GyrA domain stabilizes DNA binding to the enzyme [4]. Topoisomerase IV has been described in several bacteria and has been obtained in recombinant form from *Escherichia coli*, *Staphylococcus*

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aureus, and *Streptococcus pneumoniae* [5–8]. The enzyme has a heterotetrameric $ParC_2ParE_2$ structure, with 40–50% homology between the ParC and GyrA subunits, and between the ParE and GyrB subunits, respectively. It has been suggested that the topoisomerase IV originated by duplication of gyrase genes [9]. Topoisomerase IV cannot catalyze DNA supercoiling and has been demonstrated to be the primary decatenase in *E. coli*. Genetic studies indicate that topoisomerase IV and DNA gyrase are both essential enzymes in *E. coli* and in *Bacillus subtilis* [1–3].

Given the important role of DNA supercoiling in DNA replication, transcription, and chromosome dynamics, it is not surprising that DNA gyrase genes have been found in all bacterial genomes sequenced to date. By contrast, though topoisomerase IV genes have also been reported in most bacterial species, they are absent in a few bacteria such as *Treponema pallidum*, *Helicobacter pylori*, and notably *Mycobacterium tuberculosis*, the intracellular pathogen that causes tuberculosis [10]. Consequently, these bacteria are unusual in producing a "single" type II topoisomerase. We have recently obtained a highly purified recombinant preparation of the "single" type II topoisomerase of *M. tuberculosis* and explored its characteristics as a target for quinolones [11]. Herein, we investigated the topoisomerase functions of the *M. tuberculosis* enzyme. We found that the *M. tuberculosis* enzyme supercoils DNA with an efficiency comparable to that of other DNA gyrases, e.g., that of *E. coli*, but shows enhanced relaxation, DNA cleavage, and decatenation activities. However, the unique type II topoisomerase of *M. tuberculosis* decatenates kDNA less efficiently than a genuine topoisomerase IV, e.g., that of *S. pneumoniae*.

Materials and methods

Substrates, inhibitors, and reagents. Supercoiled plasmid pBR322 DNA was purchased from Roche (Roche Diagnostics, Meylan Cedex, France), relaxed plasmid pBR322 DNA from John Innes Enterprises, Ltd. (Norwich Research Park, Colney, Norwich, UK), and kinetoplast DNA (kDNA) from Topogen (Denver, CO, USA). The following chemicals were used: magnesium acetate and manganese chloride (Sigma–Aldrich Chimie, Saint Quentin Fallavier, France), calcium chloride (Prolabo, Paris, France), and potassium glutamate (Sigma–Aldrich Chimie). ATP, CTP, GTP, and TTP were from Sigma–Aldrich Chimie, and dATP, dTTP, dCTP, and dGTP were supplied by Eurogentec (Angers, France). All reagents were of >98% purity. ADP (adenosine 5'-diphosphate) and AMP-NP (5'-adenylylimidodiphosphate) were obtained from Sigma–Aldrich.

Gatifloxacin (Grünenthal, Levallois-Perret, France), moxifloxacin (Bayer Pharma, Puteaux, France), levofloxacin and ofloxacin (Aventis, Paris, France), and novobiocin (Sigma–Aldrich Chimie, Saint Quentin Fallavier, France) were provided by the manufacturers.

General conditions for testing topoisomerase activity of the type II topoisomerase of M. tuberculosis. Plasmids expressing *M. tuberculosis* type II topoisomerase genes were constructed as described previously [11]. Briefly, the topoisomerase genes of *M. tuberculosis* H37Rv were cloned into pET-29a (Novagen, Merck Eurolab, Fontenay-sous-Bois, France) (pATB) for the A subunit gene and pET-19b (Novagen) (pBTB) for the B subunit. Proteins were expressed and purified as previously described [11] but modifications of the protocol have been made to enhance the quantity (up to 1 mg obtained from a 500 ml culture) and the quality of the subunits tested further for enzymatic activity. These modifications are the following: (1) *E. coli* BL21-CodonPlus (λDE3)-RP cells (Stratagene, California, USA) were used as the expression host to produce separately the two subunits as His-tagged recombinant proteins, (2) in the expression experiments, the *E. coli* transformant harboring the recombinant *gyrA* plasmid (pATB) was cultured at 30 °C instead of 37 °C, (3) the Ni-NTA resin was supplied by Novagen (NOVAGEN, Merck Eurolab, Fontenay-sous-Bois, France), and (4) the total volume of the elution fraction was dialyzed overnight at 4 °C against 2.5 L of 50 mM Tris–HCl (pH 7.9) and 30 min against 1 L of 50 mM Tris–HCl (pH 7.9), and 30% glycerol.

The quantity of each subunit that was necessary for the function tested with regard to the amount of substrate and the conditions of the experiment was defined in a titration experiment where one subunit was tested with a constant quantity of the other subunit in excess.

Topoisomerase activities were tested first under classic conditions described previously for other DNA gyrases and topoisomerases IV. Second, as done for other topoisomerases (II), we defined conditions that may be preferred by the type II topoisomerase of *M. tuberculosis*. Topoisomerase activities were thus tested in different environments varying the cation used (Mg^{2+}, Ca^{2+}, and Mn^{2+}) from 0 to 10 mM, the NTPs and dNTPs used from 0 to 10 mM, and by adding potassium glutamate from 0 to 500 mM.

To facilitate direct comparison, all assays were carried out and processed with the same batch of purified subunits. All assays were done at least three times with reproducible results.

DNA supercoiling assay. For DNA supercoiling activity, the two purified subunits of the *M. tuberculosis* type II topoisomerase were added to 0.4 μg relaxed pBR322 DNA as the substrate and incubated at 37 °C for 1 h in a total volume of 30 μl. The basic DNA gyrase assay buffer was 40 mM Tris–HCl, pH 7.5, 25 mM KCl, 2 mM spermidine, 4 mM DTT, 0.1 mg/ml *E.* coli tRNA, 0.36 mg/ml BSA, 6 mM magnesium acetate, and 1 mM ATP at pH 8.0. The products were run for 5.5 h at 50 V in a 1% agarose gel and supercoiling activity was assessed by tracing the fluorescence of the bands in an ethidium-bromide stained gel corresponding to the supercoiled pBR322 DNA using Molecular Analyst®. One unit of enzyme activity was defined as the amount of each subunit that converted 0.4 μg of relaxed pBR322 to the supercoiled form in 1 h at 37 °C.

DNA relaxation. DNA relaxation was carried out as for DNA supercoiling except that 0.4 μg relaxed pBR322 was the substrate, the gyrase buffer was tested without ATP and the total reaction volume was 20 μl. After 1 h at 37 °C, reactions were stopped and analyzed by agarose gel electrophoresis as described for the DNA supercoiling assay. One unit of enzyme activity was defined as the amount of each subunit that converted 0.4 μg of supercoiled pBR322 to the relaxed form in 1 h at 37 °C.

DNA cleavage and site-specific cleavage mediated by quinolones. For DNA cleavage, the two type II topoisomerase subunits were added to 0.4 μg supercoiled pBR322 DNA as the substrate and were incubated for 1 h at 25 °C after adding fixed concentrations of breakage inducers such as quinolones and calcium. The basic buffer was the same as used for DNA supercoiling and relaxation. After incubation, 3 μl of 2% SDS and 3 μl of 1 mg/ml proteinase K were added to the reaction mixture to release DNA breaks, and incubation was continued for 30 min at 37 °C. The extent of DNA cleavage was quantified using Molecular Analyst®. Plasmid pBR322 linearized by *Eco*RI digestion was used as a marker for cleaved DNA. For cleavage activity, since the definition of units is not usually done because the cleavage varies with regard to the concentration of quinolones, we defined 1 U of cleavage activity as the amount of enzyme able to cleave 50% of 0.4 μg of supercoiled pBR322 in presence of 50 μg/ml of gatifloxacin in 1 h at 25 °C.

For the determination of site-specific DNA cleavage, the substrate was the plasmid pBR322 linearized at the *Eco*RI site, and incubation was for 1 h at 37 °C in the presence of different quinolones (moxifloxacin, ofloxacin, gatifloxacin, and levofloxacin). For this experiment, the DNA cleavage profiles obtained for the type II topoisomerase of *M. tuberculosis* were compared to those generated by the DNA gyrase and topoisomerase IV of *S. pneumoniae*. Drug concentrations were chosen so as the enzymes (*M. tuberculosis* type II topoisomerase, *S. pneumoniae* gyrase, and

S. pneumoniae topoisomerase IV, respectively) produced comparable levels of DNA breakage: 4, 15, and 20 μg/ml for moxifloxacin and for gatifloxacin, and 20, 50, and 50 μg/ml for levofloxacin and ofloxacin which have been shown to be less potent (1, 30).

Kinetoplast DNA decatenation assay. For the decatenation assay, the two purified subunits of the *M. tuberculosis* type II topoisomerase were added to 450 ng of kDNA as the substrate and incubated at 37 °C for 1 h in a total volume of 20 μl. The basic decatenation buffer was 40 mM Tris–HCl, pH 7.5, 10 mM NaCl, 6 mM magnesium acetate, 10 mM DTT, BSA 0.5 mg/ml, and 1 mM ATP (pH 8.0). The products were run for 3 h at 50 V in 1% agarose after addition of 50% glycerol–0.25% bromophenol blue and decatenation activity was assessed by tracing the fluorescence of the bands in an ethidium-bromide stained gel corresponding to the minicircles produced using Molecular Analyst®. Decatenation experiments were also performed by terminating the assay by the addition of 3 μl of 2% SDS and 3 μl of 1 mg/ml proteinase K followed by incubation for 15 min at 37 °C in order to avoid high quantities of protein loading with DNA during the agarose electrophoresis. The specific activity was determined as the amount of enzyme that produced total decatenation of 0.45 μg of kDNA in 1 h at 37 °C. Topoisomerase IV of *S. pneumoniae* was used as the reference decatenase under the same conditions [8]. DNA gyrase of *E. coli* was tested as a control for decatenation activity mediated by a DNA gyrase. For *S. pneumoniae* topoisomerase IV and *E. coli* DNA gyrase, titration was done as described above in the "*General conditions for testing topoisomerase activity of the type II topoisomerase of M. tuberculosis*" section.

Results

Intramolecular DNA processing activities of the type II topoisomerase of M. tuberculosis

The recombinant type II *M. tuberculosis* topoisomerase supercoiled relaxed pBR322 DNA gyrase with a specific activity reaching 2×10^4 U/mg (Fig. 1). Supercoiling was observed for ATP concentrations ranging from 0.5 to 8 mM, but was reduced at higher concentrations. Partial supercoiling was observed when ATP was replaced by dATP or dTTP whereas no supercoiling was observed for either CTP, GTP or other dNTPs. Mg^{2+} was the cation required for DNA supercoiling with an optimum concentration of 6 mM (Fig. 1). Supercoiling was drastically reduced when Mn^{2+} was used as the cation, and no supercoiling was observed in presence of Ca^{2+} (Fig. 1). A 10-fold increase in the supercoiling activity was observed when KGlu was added (optimum concentration of 100 mM) (data not shown). The supercoiling process was greatly inhibited by novobiocin, an ATP hydrolysis competitor, whose 50% inhibitory concentration was estimated as low as 0.5 μg/ml (Fig. 2).

Supercoiled plasmid pBR322 DNA was relaxed by the type II topoisomerase of *M. tuberculosis* with a specific activity of 10^4 U/mg (a combination of 100 ng of GyrA plus 50 ng of GyrB) (Fig. 3) and did not require ATP. Moreover, relaxation was inhibited by addition of ATP, even at low concentration (e.g., 1 mM), and ADP or AMP-NP, but not by addition of other NTPs (CTP, GTP, or TTP) or dNTPs (data not shown). Mg^{2+} was required for DNA relaxation; an increase of relaxed form of DNA was seen in the presence of Mn2+, but no relaxation was observed in presence of Ca^{2+} (Fig. 3). In contrast to DNA supercoiling, relaxation activity was inhibited by KGlu.

Mycobacterium tuberculosis type II topoisomerase was very proficient in DNA cleavage since 100 ng of GyrA plus 50 ng of GyrB was sufficient to lead to cleavable complexes stabilized by gatifloxacin revealed as linear DNA (Fig. 4). The addition of ATP, ADP or AMP-NP enhanced DNA cleavage and allowed better quantification of the cleaved bands by inhibiting DNA relaxation. Mg^{2+} was required for cleavage and its optimum concentration was 6 mM.

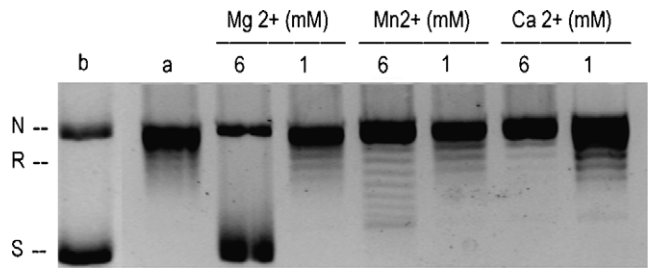

Fig. 1. DNA supercoiling activity of the type II topoisomerase of *M. tuberculosis* measured on relaxed pBR322 (0.4 μg) as the substrate and the influence of 6 and 1 mM of different cations (Mg^{2+}, Mn^{2+} or Ca^{2+}). Lanes a and b, relaxed pBR322 and DNA supercoiling of *E. coli* gyrase, respectively. N, R, and S denote nicked, relaxed, and supercoiled DNA, respectively.

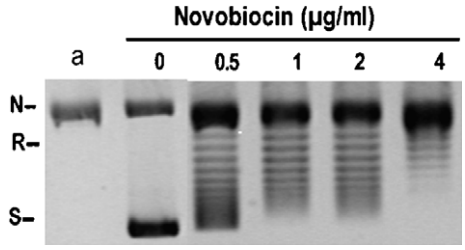

Fig. 2. Supercoiling activity of the type II topoisomerase of *M. tuberculosis* is inhibited by novobiocin. Relaxed pBR322 (0.4 μg) was incubated with the type II topoisomerase of *M. tuberculosis* in the absence and the presence of various concentrations of novobiocin (μg/ml). Lane a, relaxed pBR322. N, R, and S denote nicked, relaxed, and supercoiled DNA, respectively.

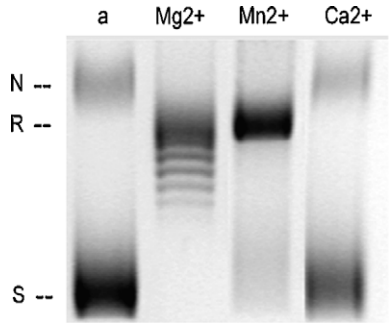

Fig. 3. DNA relaxation activity of *M. tuberculosis* type II topoisomerase measured on supercoiled pBR322 (0.4 μg) as the substrate and the influence of 6 mM of different cations (Mg^{2+}, Mn^{2+} or Ca^{2+}). Lane a, negatively supercoiled pBR322. N, R, and S denote nicked, relaxed, and supercoiled DNA, respectively.

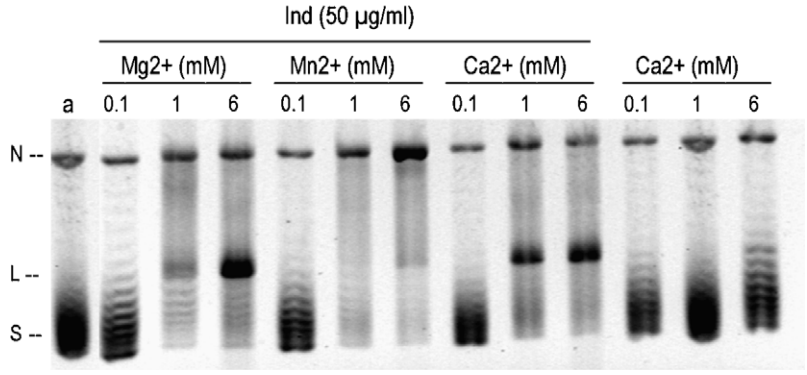

Fig. 4. DNA cleavage by the type II topoisomerase of *M. tuberculosis* measured on supercoiled pBR322 (0.4 µg) as a substrate in the presence of an inducer (Ind; gatifloxacin at 50 µg/ml) and influence of 0.1, 1, and 6 mM concentrations of different cations (Mg^{2+}, Mn^{2+}, and Ca^{2+}). Ca^{2+}-dependent DNA cleavage per se was also tested (absence of gatifloxacin). N, L, and S denote nicked, linear, and supercoiled DNA, respectively.

When Mn^{2+} replaced Mg^{2+}, nicked DNA was produced (Fig. 4) as observed in the relaxation assay. When Mg^{2+} was replaced Ca^{2+}, DNA cleavage occurred even at lower concentration (e.g., 1 mM) but not in the absence of the inducer gatifloxacin (Fig. 4). DNA cleavage activity was about 10-fold more efficient in the presence of KGlu (optimum concentration of 100 mM) (data not shown).

The site-specific DNA cleavage activity of *M. tuberculosis* type II topoisomerase and of *S. pneumoniae* gyrase and topoisomerase IV was compared using different inducers (moxifloxacin, gatifloxacin, ofloxacin, and levofloxacin). In the absence of quinolones, all the enzymes failed to induce detectable DNA cleavage. Addition of any of the four quinolones promoted enzyme-mediated DNA breakage at multiple sites. For each given enzyme, a unique DNA cleavage pattern was obtained whatever the quinolone used. For the type II topoisomerase of *M. tuberculosis*, the patterns were significantly closer to that of the DNA gyrase of *S. pneumoniae* than to that of the *S. pneumoniae* topoisomerase IV from which they were markedly different (Fig. 5).

Intermolecular DNA processing activity of the type II topoisomerase of M. tuberculosis

Decatenation activity was assessed using *Crithidia fasciculata* kinetoplast DNA (kDNA), a topologically interlocked network of DNA circles comprising ~5000 minicircles about 2.5 kb in size and 25 maxicircles of 37 kb kDNA. kDNA alone did not migrate from the wells (Fig. 6) and the topoisomerase IV of *S. pneumoniae*, tested as a reference decatenase, catalyzed decatenation resulting in total unlinking of the kDNA and exclusive production of relaxed minicircles (Fig. 6, lane SP). The use of 2 ng

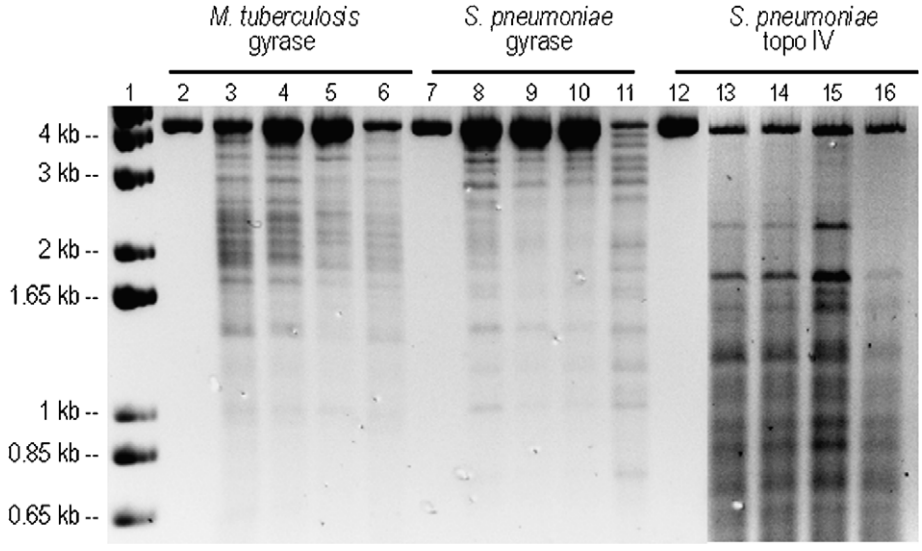

Fig. 5. Site-specific quinolone-promoted DNA cleavage by the type II topoisomerase of *M. tuberculosis*, and the DNA gyrase and the topoisomerase IV of *S. pneumoniae* (0.45 µg of GyrA or ParC and 1.7 µg of GyrB or ParE) measured on *Eco*RI-linearized pBR322 DNA (0.4 µg) as the substrate in the presence of moxifloxacin (lanes 3, 8, 13), gatifloxacin (lanes 4, 9, and 14), ofloxacin (lanes 5, 10, and 15), and levofloxacin (lanes 6, 11, and 16). Following treatment with SDS and proteinase K, the DNA samples were examined by electrophoresis in 1% agarose. Drug concentrations were chosen so as to produce comparable levels of DNA breakage. Lanes 2, 7, and 12, enzyme but no drug induced. Lane 1, size markers (sizes are indicated to the left in kb).

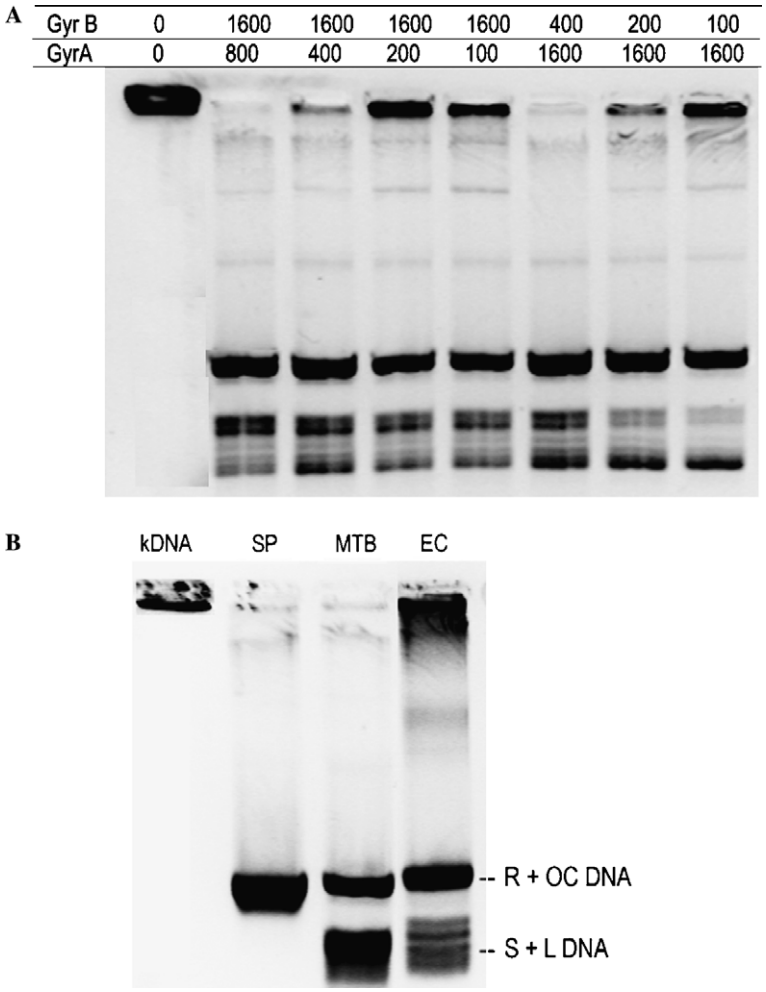

Fig. 6. (A) Titration of the kinetoplast DNA decatenation by the type II topoisomerase of *M. tuberculosis*. kDNA (0.45 μg) was incubated in reaction buffer with constant quantity of GyrA or GyrB (1600 ng) and various quantities of GyrB or GyrA as indicated (in ng) on the figure. (B) Kinetoplast DNA decatenation by the type II topoisomerase of *M. tuberculosis* compared with *S. pneumoniae* topoisomerase IV (reference topoisomerase IV) and *E. coli* DNA gyrase (reference DNA gyrase). kDNA (0.45 μg) was incubated in reaction buffer with ParC (2 ng) and ParE (20 ng) of *S. pneumoniae* topoisomerase IV (SP), with GyrA (500 ng) and GyrB (250 ng) of the type II topoisomerase of *M. tuberculosis* (MTB), or with 500 ng of reconstituted *E. coli* DNA gyrase (EC). DNA products were examined by electrophoresis in a 1% agarose gel. The position of monomer DNA circles is indicated: R DNA, relaxed monomer DNA; OC DNA, open circular DNA; L DNA, linear monomer DNA; S DNA, supercoiled monomer DNA.

ParC and 20 ng ParE proteins from *S. pneumoniae* topoisomerase IV was the minimal amount (i.e., 1 U) able to decatenate completely 0.45 μg of kDNA in 1 h at 37 °C. Under the same experimental conditions, total decatenation was obtained with 500 ng of the A subunit and 250 ng of the B subunit of the type II topoisomerase of *M. tuberculosis* as determined in the titration experiment (Fig. 6A). Comparison of the amounts of enzyme (decatenation units) showed that the *M. tuberculosis* enzyme (Fig. 6B, lane MTB) had a decatenation activity 25-fold lower than that of *S. pneumoniae* topoisomerase IV. By contrast, the same amount (500 ng) of *E. coli* DNA gyrase only partially unlinked kDNA since a large part of the kDNA remained in the well (Fig. 6B, lane EC). Decatenation by the *E. coli* gyrase remained incomplete even when higher quantities were used (3000 ng) or when the incubation time was extended (6 h) (data not shown). Based on the minimal quantity of enzyme required to produce a visible amount of DNA minicircles, the *M. tuberculosis* enzyme was estimated to be at least 10-fold more efficient in promoting kDNA decatenation than *E. coli* DNA gyrase.

As shown in Fig. 6, decatenation by *M. tuberculosis* topoisomerase released also a range of other DNA forms migrating more rapidly than relaxed and nicked minicircles and corresponding to supercoiled minicircles and linear DNA. Decatenation by *E. coli* gyrase also yielded similar products, although in lower quantities. Some of the minicircle DNA appeared to be covalently linked to protein since subsequent treatment with proteinase K resulted in predominantly nicked (also called open circular [OC] DNA) and supercoiled minicircles and less linear DNA which migrated as a doublet (Fig. 7). To determine the nature of this unusual doublet band, we included ofloxacin

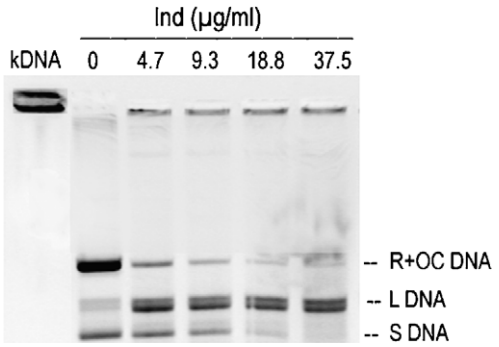

Fig. 7. KDNA decatenation by the type II topoisomerase of *M. tuberculosis* in the presence of quinolone (indicated concentrations of gatifloxacin in μg/ml) promoting the formation of cleavable complex and after additional treatment of the samples by SDS–proteinase K. DNA products were examined by electrophoresis in a 1% agarose gel. The position of monomer DNA circles is indicated: R DNA, relaxed monomer DNA; OC DNA, open circular DNA; L DNA, linear monomer DNA; S DNA, supercoiled monomer DNA.

and showed that an increase in ofloxacin concentration led to the increase of this doublet band confirming that this band corresponds to linear DNA (ofloxacin-mediated DNA breakage) (Fig. 7). Therefore, decatenation by *M. tuberculosis* type II topoisomerase led to accumulation of covalent DNA enzyme intermediates producing linear and nicked DNA, unlike *S. pneumoniae* topoisomerase IV.

For decatenation, like *S. pneumoniae*, *M. tuberculosis* type II topoisomerase required ATP (minimal concentration 0.5 mM) and Mg^{2+} (concentration between 6 and 10 mM). Finally, a relatively high concentration (250 mM) of KGlu improved 10-fold the decatenation activity of *M. tuberculosis* enzyme (data not shown).

Topoisomerase activities (supercoiling, relaxation, cleavage, and decatenation) exhibited by the *M. tuberculosis* enzyme were compared and were expressed as relative activities based on the reference activity of double-strand breakage. Globally, the type II topoisomerase of *M. tuberculosis* appeared as a polyvalent enzyme with only a four-fold difference between the amount required to assess the four topoisomerase functions that we explored (Table 1).

Discussion

Mycobacterium tuberculosis and a few other bacteria harbor only one type II topoisomerase in contrast with *E. coli* and other common bacteria which harbor two type II topoisomerases, DNA gyrase and topoisomerase IV. This situation raises interesting questions as to the nature of the activities of the single type II topoisomerases, and the present study is the first characterization of such an enzyme. Since, we recently succeeded in producing highly purified recombinant type II topoisomerase of *M. tuberculosis* [11], we explored the different topoisomerase capacities of this enzyme. We showed that this enzyme is as efficient as DNA gyrases from common bacteria in DNA supercoiling but more efficient than the latter in other DNA strand passage activities, such as intramolecular DNA relaxation and intermolecular decatenation as well as DNA cleavage.

The recombinant type II topoisomerase of *M. tuberculosis* was at least as efficient for DNA supercoiling as the native enzymes of *Mycobacterium smegmatis* and *Mycobacterium avium* purified in our laboratory [12,13] and in others [14], and as the recombinant His-tagged enzyme of *Mycobacterium bovis* BCG, a strain which also belongs to the same species group as *M. tuberculosis* [15]. Previous studies on recombinant topoisomerases have shown that His-tags do not hamper the function nor the interaction of topoisomerase with drugs, and that they allow a rapid and safe purification method for slow grower and pathogenic bacteria such as *M. tuberculosis* (avoiding large-scale cultures) [7,8,16].

Magnesium cations were shown to be necessary for all topoisomerase functions of the *M. tuberculosis* type II topoisomerase as described for topoisomerases from other bacteria.

Mycobacterium tuberculosis type II topoisomerase mediated ATP-independent DNA relaxation activity like DNA gyrases from common bacteria, but unlike topoisomerases IV and eukaryotic topoisomerase II that require ATP for relaxation, ATP or analogs inhibited DNA relaxation mediated by the *M. tuberculosis* enzyme. This suggested that turning the ATP binding site into a closed conformation that leads to the dimerization of the GyrB subunits [17] hampers the reverse passive strand transfer required for relaxation. The *M. tuberculosis* enzyme is very efficient for relaxation since this function needed only twice the amount of protein necessary for supercoiling whereas this ratio is 50 for DNA gyrases from common bacteria [3,7,8,18]. The *M. tuberculosis* enzyme was also able to cleave DNA with a smaller amount of enzyme than observed for other type II topoisomerases [3,8]. When we

Table 1
Activities and relative activities of the type II topoisomerase of *M. tuberculosis*

Topoisomerase functions	Specific activities[a] (U/mg) (optimal [KGLU])	Relative activities[b]
Supercoiling	2×10^4 (100 mM)	2
Relaxation	10^4 (0 mM)	1
Double-strand cleavage	10^4 (100 mM)	1
Decatenation	2×10^3 (250 mM)	0.5

[a] Activities measured in the standard experimental conditions (see text) with the indicated KGlu concentration.
[b] The activity of double-strand DNA cleavage was taken as the reference for type II topoisomerase and was attributed a value of 1.

explored the site-specific quinolone-promoted cleavage, the cleavage pattern mediated by the type II topoisomerase of *M. tuberculosis* was close to that mediated by the DNA gyrase of *S. pneumoniae* and was different from that produced by topoisomerase IV of *S. pneumoniae* (Fig. 5). As described by Leo et al. [19], differences in the quinolone structure may affect antibiotic potency but do not affect the site specificity of DNA breakage. Altogether, these results show that the type II topoisomerase of *M. tuberculosis* is an efficient gyrase in mediating intramolecular DNA events with unusually high efficiency for relaxation and cleavage. Like *E. coli* DNA gyrase and unlike topoisomerase IV, *M. tuberculosis* type II topoisomerase is inhibited by low concentrations of novobiocin. So the single type II topoisomerase of *M. tuberculosis* may be a target for coumarin drugs, as previously demonstrated for quinolones [11].

Our final interest was to assess if the *M. tuberculosis* enzyme, as a single type II topoisomerase, exhibits also the decatenase function mediated by topoisomerase IV in bacteria that harbor both enzymes. It has been shown that gyrases are relatively poor decatenases, a fact viewed as an inevitable consequence of their preference for intramolecular strand passage required for DNA supercoiling [3,20]. After the discovery of bacterial topoisomerase IV and eukaryotic topoisomerase II, decatenation and supercoiling assays were used to discriminate between the supercoiling-proficient type II topoisomerase (DNA gyrase) and supercoiling-deficient ones (eukaryotic topoisomerase II and topoisomerase IV). In support of the idea that single type II topoisomerase should be able to perform all functions attributed to gyrase and topoisomerase IV [21], we found that *M. tuberculosis* DNA gyrase has a markedly higher decatenase activity than *E. coli* DNA gyrase, and that it is able to fully decatenate the kDNA network, in contrast to the *E. coli* DNA gyrase which cannot even with high amounts of enzyme. This is similar to what has been reported on the decatenation activity of the corresponding enzyme of *M. smegmatis* [18]. However, in the *M. smegmatis* study [18], no comparison was made against the activity of a topoisomerase IV. In the present study, we used *S. pneumoniae* topoisomerase IV as a reference decatenase [3,8], and showed that the *M. tuberculosis* enzyme is on the one hand more efficient than the DNA gyrase from *E. coli*, and on the other hand is not as efficient as topoisomerase IV from *S. pneumoniae* for decatenation (Fig. 6A). These findings suggest that much care must be taken when assessing the decatenase activities of gyrase. Even if the *M. tuberculosis* gyrase has high decatenation activity, compared to the *E. coli* gyrase, it may not have sufficient biological relevance. Mycobacteria may have evolved other mechanisms than topoisomerase IV to decatenate DNA. Jain et al. described recently in *M. smegmatis* a second type II topoisomerase, topoNM, which has a potent decatenase activity [22]. However, homologs to topoNM genes do not exist in the *M. tuberculosis* genome, nor do genes homologous to those of the topoisomerase VI, which might help in the decatenation process [2]. Since *M. smegmatis* is a rapidly growing mycobacteria, it may have required a dedicated decatenating enzyme to avoid chromosome partitioning defects [21] whereas the needs of *M. tuberculosis* which is a slow growing mycobacteria may be different. Type IA topoisomerases, such as topoisomerase III, or some recombinase resolvase systems may be able to undertake decatenation [1]. However, *M. tuberculosis* has also only one type I topoisomerase, and thus lacks topoisomerase III. We may hypothesize that if DNA replication in *M. tuberculosis* is slow, positive supercoils might be removed very efficiently by this unique type II topoisomerase before they become precatenates.

Structural and biochemical studies suggest that the specificity of the intra (supercoiling) and intermolecular (decatenation) activities of a bacterial type II topoisomerase is related to the C-terminal domain of GyrA (ParC), which has been recently described as a beta-propeller or a pinwheel [2,4,23]. Indeed, the *E. coli* $A59_2B_2$ gyrase complex (in which the C-terminal GyrA domain [CTD] is deleted) decatenates 30-fold more efficiently than intact DNA gyrase [3,24]. It has been suggested that salts, such as potassium chloride or potassium glutamate, modulate the DNA binding mode of the type II topoisomerase by affecting the disposition of the GyrA-CTD to favor trapping of the T segment [3,25,26]. For the *M. tuberculosis* enzyme, we showed that KGlu was required for decatenation whereas decatenation by common DNA gyrase is known to be inhibited by KGlu [6,27]. Thus, in that aspect the *M. tuberculosis* DNA enzyme behaves more like a topoisomerase IV. Crystallographic analyses and recent studies suggest that high KGlu concentrations may destabilize the GyrA-CTD or induce a conformational change that favors intermolecular binding of DNA instead of intramolecular DNA binding [2,23,28].

The resolution of the kDNA network by *M. tuberculosis* topoisomerase II seems to be unusual. Indeed, in addition to supercoiled minicircles, a substantial amount of cleaved DNA bearing covalently linked protein was obtained, whereas only relaxed minicircles were released by *S. pneumoniae* topoisomerase IV. Protein-linked DNA products by the *M. tuberculosis* enzyme suggest that the DNA breakage–reunion equilibrium is more in favor of broken DNA. Unlinking of the kDNA network may be the ultimate result of successive processing by *M. tuberculosis* topoisomerase II that includes decatenation, cleavage, and supercoiling. This multiple process may operate because the *M. tuberculosis* enzyme produces substantial levels of each activity under the decatenation conditions, contrary to what is observed for other DNA gyrases or topoisomerases IV.

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References

[1] J.J. Champoux, DNA topoisomerases: structure, function, and mechanism, Annu. Rev. Biochem. 70 (2001) 369–413.
[2] K.D. Corbett, J.M. Berger, Structure, molecular mechanisms, and evolutionary relationships in DNA topoisomerases, Annu. Rev. Biophys. Biomol. Struct. 33 (2004) 95–118.
[3] C. Levine, H. Hiasa, K.J. Marians, DNA gyrase and topoisomerase IV: biochemical activities, physiological roles during chromosome replication, and drug sensitivities, Biochem. Biophys. Acta 1400 (1998) 29–43.
[4] K.D. Corbett, R.K. Shultzaberger, J.M. Berger, The C-terminal domain of DNA gyrase A adopts a DNA-bending beta-pinwheel fold, Proc. Natl. Acad. Sci. USA 101 (2004) 7293–7298.
[5] J. Kato, Y. Nishimura, R. Imamura, H. Niki, S. Hiraga, H. Suzuki, New topoisomerase essential for chromosome segregation in *E. coli*, Cell 63 (1990) 393–404.
[6] H. Peng, K.J. Marians, *Escherichia coli* topoisomerase IV. Purification, characterization, subunit structure, and subunit interactions, J. Biol. Chem. 268 (1993) 24481–24490.
[7] F. Blanche, B. Cameron, F.X. Bernard, L. Maton, B. Manse, L. Ferrero, N. Ratet, C. Lecoq, A. Goniot, D. Bisch, J. Crouzet, Differential behaviors of *Staphylococcus aureus* and *Escherichia coli* type II DNA topoisomerases, Antimicrob. Agents Chemother. 40 (1996) 2714–2720.
[8] X.S. Pan, L.M. Fisher, *Streptococcus pneumoniae* DNA gyrase and topoisomerase IV: overexpression, purification, and differential inhibition by fluoroquinolones, Antimicrob. Agents Chemother. 43 (1999) 1129–1136.
[9] W.M. Huang, Bacterial diversity based on type II DNA topoisomerase genes, Annu. Rev. Genet. 30 (1996) 79–107.
[10] S.T. Cole, R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S.V. Gordon, K. Eiglmeier, S. Gas, C.E. Barry 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B.G. Barrell, et al., Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence, Nature 393 (1998) 537–544.
[11] A. Aubry, X.S. Pan, L.M. Fisher, V. Jarlier, E. Cambau, *Mycobacterium tuberculosis* DNA gyrase: interaction with quinolones and correlation with antimycobacterial drug activity, Antimicrob. Agents Chemother. 48 (2004) 1281–1288.
[12] V. Revel-Viravau, Q.C. Truong, N. Moreau, V. Jarlier, W. Sougakoff, Sequence analysis, purification, and study of inhibition by 4-quinolones of the DNA gyrase from *Mycobacterium smegmatis*, Antimicrob. Agents Chemother. 40 (1996) 2054–2061.
[13] I. Guillemin, W. Sougakoff, E. Cambau, V. Revel-Viravau, N. Moreau, V. Jarlier, Purification and inhibition by quinolones of DNA gyrases from *Mycobacterium avium*, *Mycobacterium smegmatis* and *Mycobacterium fortuitum* bv. *peregrinum*, Microbiology 145 (1999) 2527–2532.
[14] K. Madhusudan, V. Nagaraja, *Mycobacterium smegmatis* DNA gyrase: cloning and overexpression in *Escherichia coli*, Microbiology 141 (1995) 3029–3037.
[15] L.C. Wu, S.I. Shahied, Mycobacterial DNA gyrase: enzyme purification and characterization of supercoiling activity, Arch. Biochem. Biophys. 324 (1995) 123–129.
[16] A.Y. Saiki, L.L. Shen, C.M. Chen, J. Baranowski, C.G. Lerner, DNA cleavage activities of *Staphylococcus aureus* gyrase and topoisomerase IV stimulated by quinolones and 2-pyridones, Antimicrob. Agents Chemother. 43 (1999) 1574–1577.
[17] V. Lamour, L. Hoermann, J.M. Jeltsch, P. Oudet, D. Moras, An open conformation of the *Thermus thermophilus* gyrase B ATP-binding domain, J. Biol. Chem. 277 (2002) 18947–18953.
[18] U.H. Manjunatha, M. Dalal, M. Chatterji, D.R. Radha, S.S. Visweswariah, V. Nagaraja, Functional characterisation of mycobacterial DNA gyrase: an efficient decatenase, Nucleic Acids Res. 30 (2002) 2144–2153.
[19] E. Leo, K.A. Gould, X.S. Pan, G. Capranico, M.R. Sanderson, M. Palumbo, L.M. Fisher, Novel symmetric and asymmetric DNA scission determinants for *Streptococcus pneumoniae* topoisomerase IV and gyrase are clustered at the DNA breakage site, J. Biol. Chem. 280 (2005) 14252–14263.
[20] J.C. Marini, K.G. Miller, P.T. Englund, Decatenation of kinetoplast DNA by topoisomerases, J. Biol. Chem. 255 (1980) 4976–4979.
[21] K.D. Corbett, A.J. Schoeffler, N.D. Thomsen, J.M. Berger, The structural basis for substrate specificity in DNA topoisomerase IV, J. Mol. Biol. 351 (2005) 545–561.
[22] P. Jain, V. Nagaraja, An atypical type II topoisomerase from *Mycobacterium smegmatis* with positive supercoiling activity, Mol. Microbiol. 58 (2005) 1392–1405.
[23] Y. Qi, J. Pei, N.V. Grishin, C-terminal domain of gyrase A is predicted to have a beta-propeller structure, Proteins 47 (2002) 258–264.
[24] S.C. Kampranis, A. Maxwell, Conversion of DNA gyrase into a conventional type II topoisomerase, Proc. Natl. Acad. Sci. USA 93 (1996) 14416–14421.
[25] J. Roca, Varying levels of positive and negative supercoiling differently affect the efficiency with which topoisomerase II catenates and decatenates DNA, J. Mol. Biol. 305 (2001) 441–450.
[26] J.G. Heddle, S. Mitelheiser, A. Maxwell, N.H. Thomson, Nucleotide binding to DNA gyrase causes loss of DNA wrap, J. Mol. Biol. 337 (2004) 597–610.
[27] K. Hoshino, A. Kitamura, I. Morrissey, K. Sato, J. Kato, H. Ikeda, Comparison of inhibition of *Escherichia coli* topoisomerase IV by quinolones with DNA gyrase inhibition, Antimicrob. Agents Chemother. 38 (1994) 2623–2627.
[28] L. Costenaro, J.G. Grossmann, C. Ebel, A. Maxwell, Small-angle X-ray scattering reveals the solution structure of the full-length DNA gyrase a subunit, Structure (Camb) 13 (2005) 287–296.