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An atypical type II topoisomerase from *Mycobacterium smegmatis* with positive supercoiling activity

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Summary

Topoisomerases are essential ubiquitous enzymes, falling into two distinct classes. A number of eubacteria including *Escherichia coli*, typically contain four topoisomerases, two type I topoisomerases and two type II topoisomerases viz. DNA gyrase and topoisomerase IV. In contrast several other bacterial genomes including mycobacteria, encode for one type I topoisomerase and a DNA gyrase. Here we describe a new type II topoisomerase from *Mycobacterium smegmatis* which is different from DNA gyrase or topoisomerase IV in its characteristics and origin. The topoisomerase is distinct with respect to domain organization, properties and drug sensitivity. The enzyme catalyses relaxation of negatively supercoiled DNA in an ATP-dependent manner and also introduces positive supercoils to both relaxed and negatively supercoiled substrates. The genes for this additional topoisomerase are not found in other sequenced mycobacterial genomes and may represent a distant lineage.

Introduction

Topoisomerases are the ubiquitous enzymes responsible for maintaining the topological state of cellular DNA. The enzymes perform various reactions on DNA including DNA relaxation, supercoiling, decatenation and unknotting and thus are involved in essential cellular processes including transcription, replication, chromosome condensation and segregation (Wang, 1991; Bates and Maxwell, 1993; Champoux, 2001). DNA topoisomerases fall into two general classes, type I and type II, which are distinguished by their ability to cleave one or both strands of DNA duplex, respectively, during a typical topoisomerase reaction involving DNA breakage, strand passage and religation. In *Escherichia coli* two type I – topoisomerase I and topoisomerase III and two type II topoisomerases – DNA gyrase and topoisomerase IV have been characterized. Topoisomerase I catalyses relaxation of negatively supercoiled DNA whereas DNA gyrase introduces negative supercoils into DNA and both the enzymes act as principal swivels during transcription (Champoux, 2001; Espeli and Marians, 2004). Topoisomerase IV and topoisomerase III possess DNA relaxation and decatenation activity (Peng and Marians, 1993; Nurse *et al*., 2003). Between the two enzymes, topoisomerase IV is the major decatenase in the cell and is essential for chromosome segregation in *E. coli* (Kato *et al*., 1990; Levine *et al*., 1998). Thus, each one of the topoisomerases has distinct roles in *E. coli* and mutation in any of the topoisomerase leads to a compromised growth phenotype (Menzel and Gellert, 1983; Kato *et al*., 1990; Schofield *et al*., 1992; Wang *et al*., 2002).

Many eubacteria do not encode for all the four topoisomerases described above. Organisms including *Mycobacterium tuberculosis* possess only one topoisomerase each of type I and II classes, i.e. topoisomerase I and DNA gyrase respectively (Cole *et al*., 1998). Such an organization, thus, appears to represent the minimal topoisomerase requirement for a bacterial cell, to tackle all the cellular topological requirements. Although most members of the genus mycobacteria have the same minimal topoisomerase content (Philipp *et al*., 1996; Cole *et al*., 2001; Camus *et al*., 2002; Fleischmann *et al*., 2002; Garnier *et al*., 2003), analysis of *Mycobacterium smegmatis* genome revealed that it has additional open reading frames (ORFs) encoding for another GyrB and a type II topoisomerase, other than DNA gyrase. In this manuscript the studies on the additional type II topoisomerase in *M. smegmatis* are described. Results reveal that the enzyme is not a typical topoisomerase IV and exhibits properties which are of a distinct type II topoisomerase hitherto not described.

Results

Additional type II topoisomerase in M. smegmatis

Two ORFs of 698 and 713 amino acids, named TopoN and TopoM (TIGR locus: MSMEG0448 and MSMEG0447, respectively), were identified in *M. smegmatis* genome sequence with a similarity of 37.5% and 37.8% to its GyrB and GyrA subunits respectively. The similarity scores were

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even lower, 23% and 21%, respectively, when compared with GyrB and GyrA from other bacteria. Furthermore, TopoN and TopoM also showed a very low degree of similarity (24% and 21%, respectively) to ParE and ParC from various bacteria. In contrast to this observation, the multiple sequence alignment of type II topoisomerase sequences of eubacteria show a high degree of homology (Caron and Wang, 1994). Generally, gyrase and topoisomerase IV genes from different species show very high degree of similarity (50–70%) to the respective genes from other species of bacteria. Moreover, within a given species, the genes for DNA gyrase show significant similarity with their topoisomerase IV alleles (40–55%). Against this background, a low degree of similarity of TopoNM with either gyrase subunits or topoisomerase IV subunits is not a typical feature. In spite of significant variation in the sequence alignment with other type II topoisomerases, the two ORFs (TopoN and TopoM) appeared to retain the conserved catalytic motifs characteristic of typical type II topoisomerases suggesting that the two genes might encode for the subunits of a type II topoisomerase, functioning together as in the case of other enzymes of this class (Fig. 1A).

Phylogenetic analysis of TopoNM

The two typical type II topoisomerases, DNA gyrase and topoisomerase IV, belong to type IIA class. They are distinguished on the basis of their biochemical properties and distinct intracellular roles (Espeli and Marians, 2004). To evaluate the origin and evolutionary relationship of TopoNM with other type IIA topoisomerases, phylogenetic analysis was carried out by comparing full-length sequences of TopoN with ParE sequences and TopoM with ParC and GyrA sequences respectively (Fig. 1B and C). The evolutionary tree was also constructed by using the ATPase domain of TopoN, a region well conserved in type II topoisomerases (Fig. 1D). All the three phylogenetic trees showed a very distant relationship of TopoNM subunits to that of other type IIA topoisomerases, indicating an early divergence of TopoNM.

Characterization of TopoNM

The *topoN* and *topoM* were cloned and expressed in *E. coli.* Purified TopoN and TopoM proteins have the predicted sizes of 74 and 77 kDa respectively (Fig. 2A). Individual polypeptides do not exhibit relaxation or supercoiling activity while the reconstituted enzyme, comprising of both the subunits (TopoNM), catalysed the relaxation of negatively supercoiled DNA in presence of ATP (Fig. 2B). The specific activity of the enzyme was determined by titration of individual subunits against each other and found to be 2.25×10^4 U mg^{-1} and 6.49×10^4 U mg^{-1} for TopoN and TopoM proteins respectively. One unit of TopoNM comprised of 30 nM of TopoN and 10 nM of TopoM subunits (Fig. 2B). The Negative supercoiling activity, characteristic of DNA gyrase, was not observed in spite of highest similarity to DNA gyrase (37%) of *M. smegmatis* (not shown). The DNA relaxation activity of the enzyme is not stimulated by potassium glutamate in contrast to that of *M. smegmatis* DNA gyrase (Chatterji *et al.,* 2001). The relaxation activity was observed only in presence of ATP/dATP and the enzyme was inactive in presence of any other NTP/dNTPs or a non-hydrolysable analogue of ATP (Fig. 2C). Activity of TopoNM was magnesium-dependent (Fig. 2D) and was inhibited above 150 mM NaCl (Fig. 2E). Furthermore, gel filtration analysis was carried out to investigate the oligomeric status of the enzyme. TopoNM activity was reconstituted by incubating TopoN and TopoM in a molar ratio of 3:1 as determined in Fig. 2B. The TopoN, TopoM and TopoNM elution profiles were analysed in a Superdex S-200 molecular sieving column. The protein peaks corresponding to TopoN, TopoM and TopoNM were observed at 16.5 ml, 15.2 ml and 13.7 ml corresponding to the molecular masses of 80 kDa, 145 kDa and 282 kDa respectively. From these data the Stokes radii of TopoN, TopoM and TopoNM were calculated to be 40.2 Å, 51 Å and 63.4 Å respectively (Fig. S1). These results indicate that TopoNM is a heterotetramer in solution like other eubacterial type II topoisomerases. These data also show TopoN to be a monomer by itself while TopoM is a dimer in solution (Fig. S1)

Results presented above suggest that TopoNM is a type II topoisomerase. Another hallmark feature of type II enzymes is the generation of transient double strand breaks in DNA before strand passage and religation steps. Double strand cleavage is visualized by trapping the enzyme-DNA covalent intermediate using quinolone class of compounds (Heddle *et al.*, 2001). Supercoiled pUC18 DNA was incubated with the enzyme and ciprofloxacin and the reactions were arrested by the addition of SDS followed by proteinase K digestion. Formation of linear DNA by TopoNM in these reactions confirmed that the enzyme is a type II topoisomerase (Fig. 2F, lane 3–8). Substitution of calcium in place of magnesium in DNA relaxation assays resulted in the generation of double strand breaks in DNA (data not shown).

Introduction of positive supercoils by TopoNM

When relaxation assays were carried out using 0.5–3.0 units of TopoNM, few additional topoisomers appeared at enzyme units higher than 1.5 (Fig. 3A lane 4–7). TopoNM also introduced additional topoisomers into the relaxed DNA substrate (Fig. 3B lane 3–7). To determine the nature of these topoisomers, the products of the relaxation

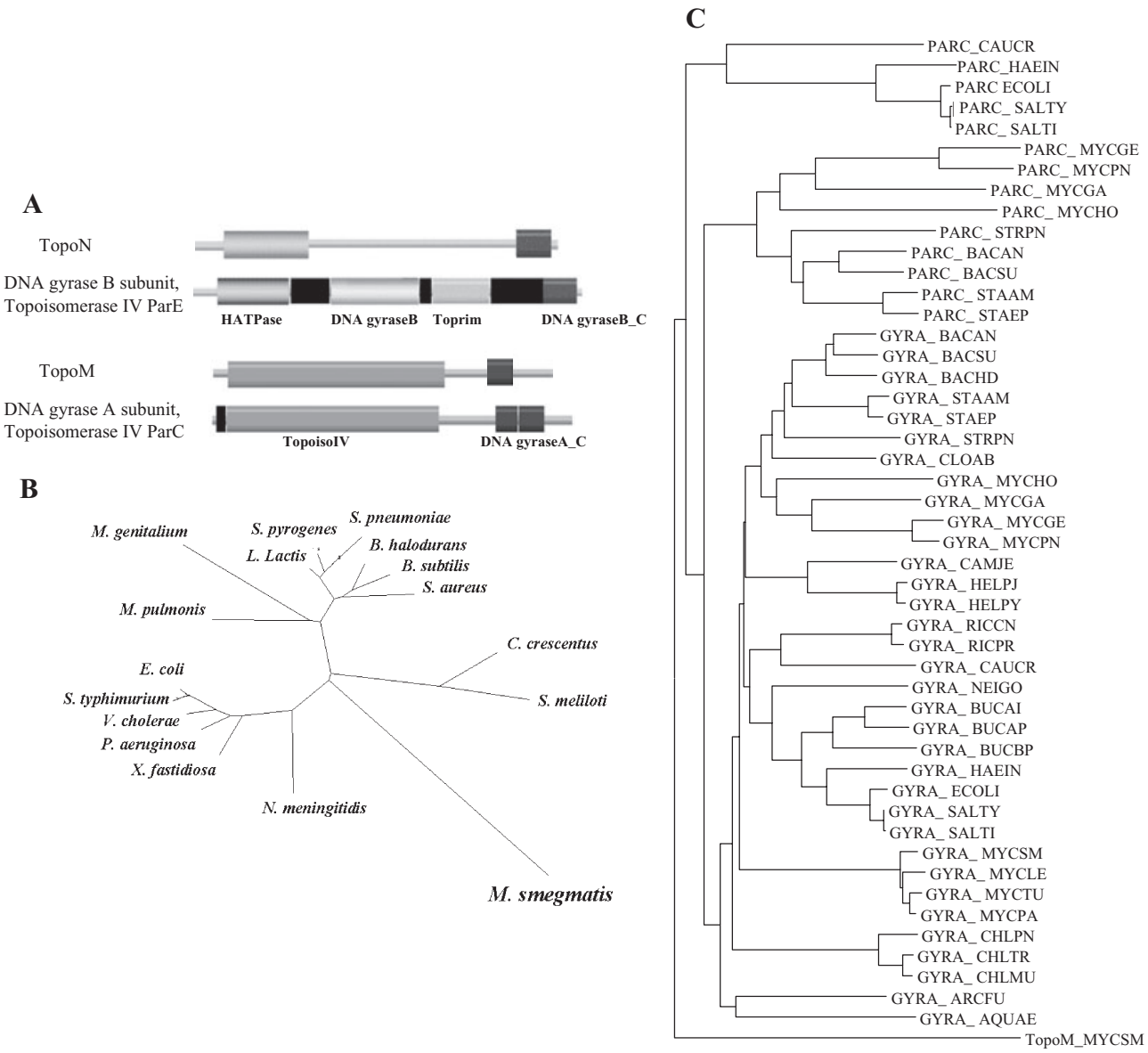

Fig. 1. Phylogenetic analysis of TopoNM.
A. Domain organization of type II topoisomerases. Comparison of various domains present in TopoN of *M. smegmatis* with other eubacterial ParE and GyrB. Functional domains identified by pfam are indicated. Two conserved domains of type II enzymes namely, Toprim and DNA gyraseB domain could not be identified in TopoN. In TopoM subunit, both topoisoIV and gyrA_C, the domains present in eubacterial ParC and GyrA, were identified.
B. Phylogenetic tree of TopoN. Phylogenetic tree deduced from amino acid sequences of TopoN of *M. smegmatis* and ParE from other eubacteria.
C. Phylogenetic comparison of TopoM with GyrA and ParC. Phylogenetic tree deduced from amino acid sequences of TopoM of *M. smegmatis* and GyrA and ParC from other eubacteria.
D. Phylogenetic tree deduced from amino acid sequences of ATPase domain of TopoN and other type II topoisomerases. The species names are listed according to the swiss-prot code. Sequence alignments were produced by CLASTALW. Phylogenetic trees were generated with MEGA2 using the neighbour-joining algorithm.

assays were resolved both in presence and absence of ethidium bromide (EtBr) (Fig. 4). At 0.25 and 0.5 enzyme units, migration of topoisomers was retarded in presence of EtBr indicating negative superhelicity of DNA (compare lanes 2 or 3 of Fig. 4A with 4B). In contrast, the topoisomers formed at 1.5 and 1.75 enzyme units migrated faster in the presence of EtBr indicating positive superhelicity of the DNA (compare lane 7 or 8 of Fig. 4A and B). For further confirmation, assays were carried out at three different enzyme concentrations (0.5 U, 1.5 U and 2.5 U) and the products were resolved by two-dimensional agarose gel electrophoresis (Fig. 5A). At 0.5 units of

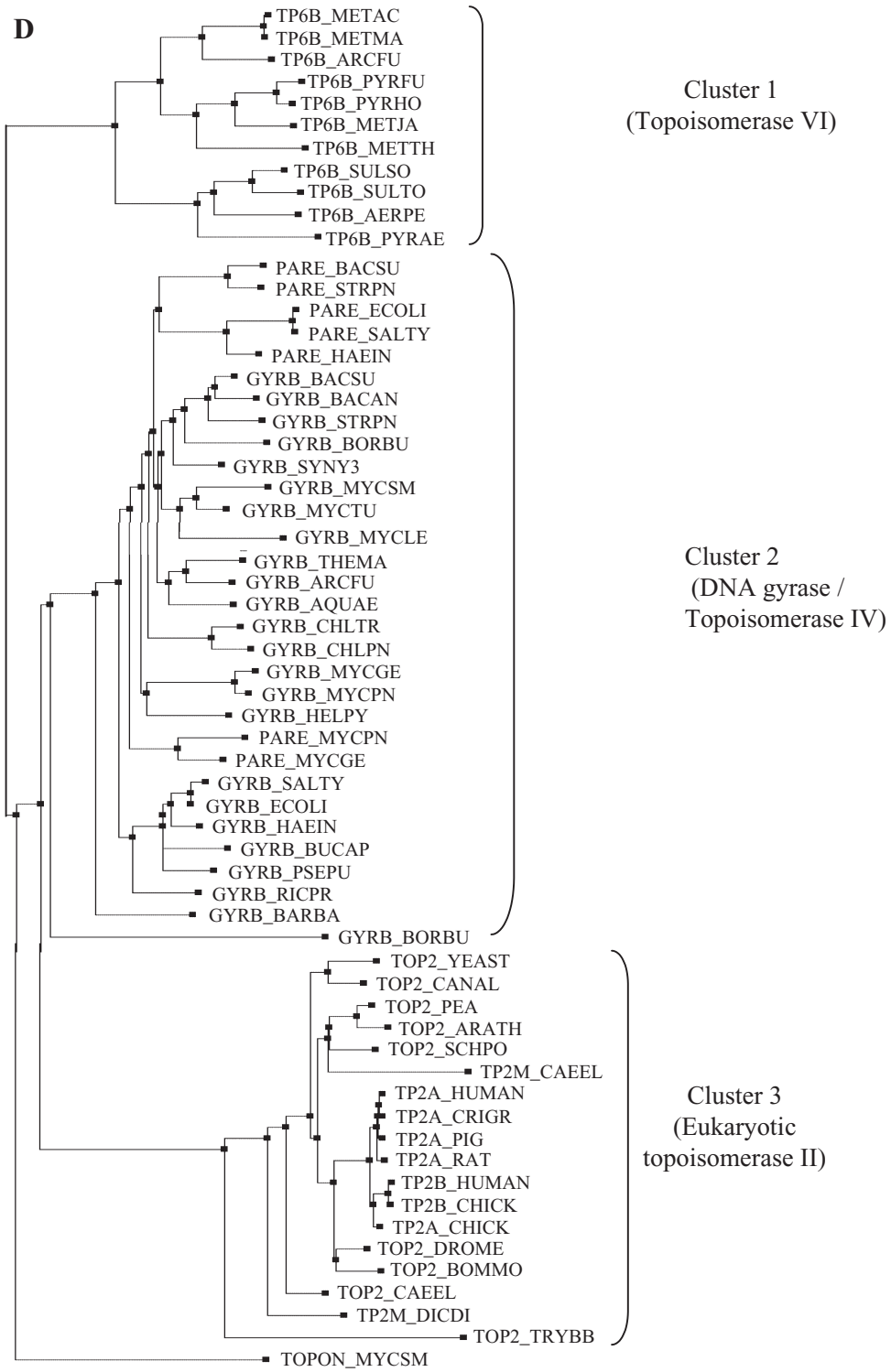

Fig. 1. *cont.*

enzyme, when DNA relaxation by TopoNM is not complete, a left-hand arc is formed, characteristic of negative supercoils in a two-dimensional gels (Fig. 5B, lane 2). At 2.5 units of TopoNM, topoisomers showed a mobility of right-hand diagonal and thus were positively supercoiled (Fig. 5B, lane 4). Assays carried out in parallel, with different amounts of topoisomerase I and DNA gyrase of *M. smegmatis* showed the respective DNA relaxation or

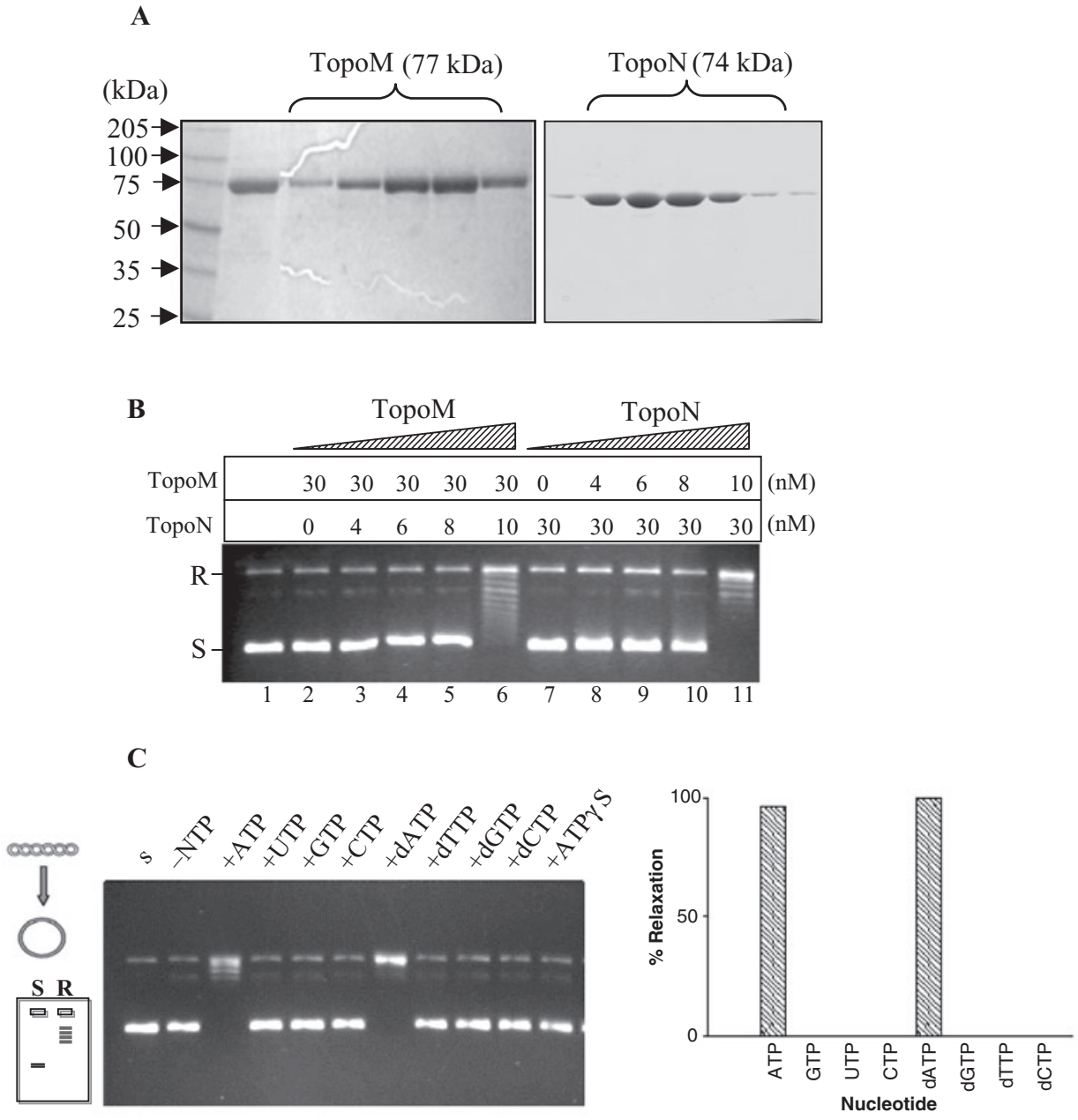

Fig. 2. Purification of TopoNM and activity assays.
A. Purified fractions of TopoN and TopoM subunits after respective monoQ column chromatography.
B. Reconstitution of enzyme activity. Activity of TopoNM was determined by titration of TopoM with TopoM and vice versa. Lane 1: pUC18 DNA substrate. Lane 2: DNA with TopoM. Lanes 3–6: Titration of TopoM with TopoN. Lane 7: DNA with TopoN. Lanes 8–11: Titration of TopoN with TopoM.
C. Activity of TopoNM in presence of various energy cofactors.
D and E. Effect of magnesium (D) and ionic strength (E) on TopoNM activity.
F. TopoNM generates double-stranded breaks in DNA in presence of ciprofloxacin. Lane 1: pUC18 DNA substrate. Lane 2. DNA with 20 units of TopoNM, Lanes 3–8: DNA with TopoNM and 0.1, 0.2, 0.5, 1.0, 1.5 and 2.0 mg ml^{-1} of ciprofloxacin. Position of linear DNA is indicated by arrowhead. S represents negatively supercoiled DNA. R represents relaxed DNA.

supercoiling activity; topoisomers with the positive supercoils were not observed (Fig. 5C and D).

The introduction of positive supercoils by TopoNM could be either due to enzymatic activity itself or wrapping of the DNA by the protein. The DNA wrapping mediated positive supercoiling by the enzyme was examined in a reaction where *M. smegmatis* topoisomerase I was used to relax the negatively supercoiled DNA in presence of TopoNM.

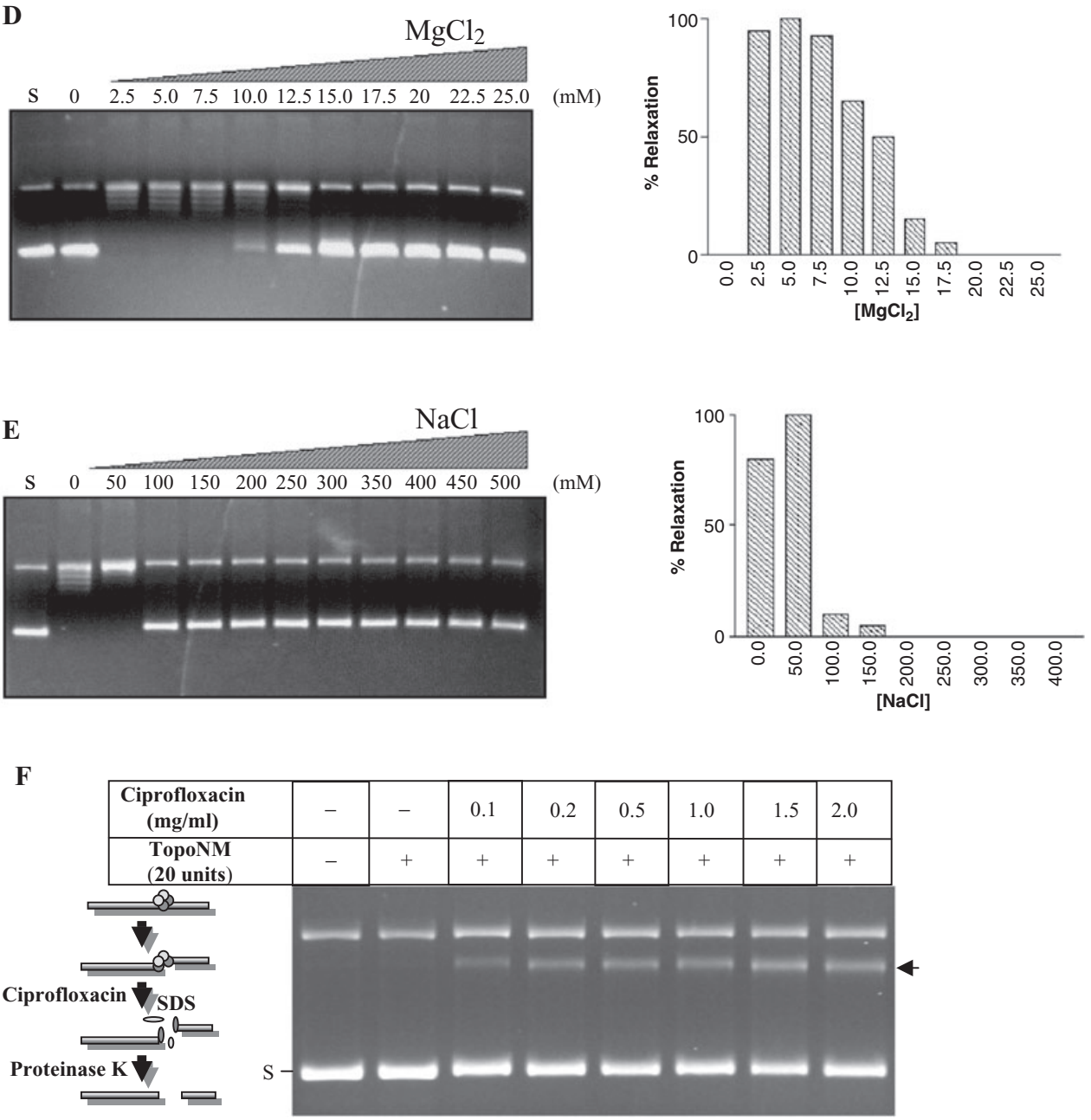

Fig. 2. *cont.*

If the compensatory negative supercoils are generated as a result of positive supercoils induced by TopoNM-mediated wrapping of DNA, these would be relaxed by topoisomerase I in absence of ATP which will result in change in DNA topology (net positive supercoils) after the removal of proteins. Absence of any topological changes in the DNA in such an experiment suggests that the enzyme is catalytically proficient in introducing positive supercoils into DNA (Fig. S2).

Relaxation of positively supercoiled DNA

Furthermore, the activity of TopoNM on positively supercoiled DNA was determined. The positively supercoiled substrate was generated by the addition of EtBr to relaxed pUC18 DNA and was then incubated with increasing concentrations of TopoNM. The EtBr was removed from the assays by butanol extraction and the reaction products were analysed by agarose gel electrophoresis. Increase

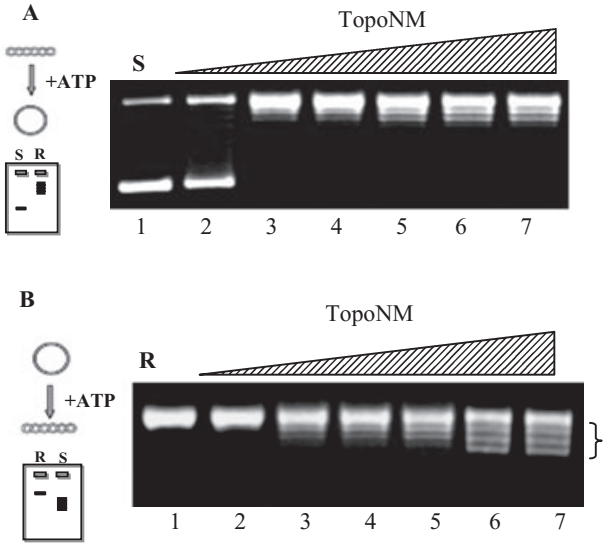

Fig. 3. Supercoiling and relaxation activity of TopoNM.
A. TopoNM relaxes negatively supercoiled DNA in the presence of ATP. Relaxation assays were carried out using 500 ng of negatively supercoiled DNA (S) and increasing concentrations of TopoNM. Lane 1: pUC18 DNA substrate. Lanes 2–7: DNA with 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 units of TopoNM. Note the appearance of additional topoisomers at higher enzyme units (lanes 5–7).
B. TopoNM also introduces supercoils in relaxed DNA. Supercoiling assays were carried out using 500 ng of relaxed pUC18 DNA (R) and increasing concentrations of TopoNM. Lane 1: relaxed pUC18 DNA. Lanes 2–7: relaxed DNA with 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 units of TopoNM. The additional topoisomers appeared have been indicated.

in the mobility of DNA in presence of TopoNM and ATP indicates that the enzyme catalyses relaxation of positively supercoiled DNA (Fig. 6). The relaxation of positively supercoiled DNA thus formed is ATP-dependent. ATP-independent relaxation of positive supercoils is not observed (Fig. S3). This property is in contrast to that of DNA gyrase, whose DNA relaxation activity is ATP-independent.

Catenation and decatenation activity of TopoNM

Next, the ability of TopoNM to catalyse inter molecular reactions was determined using either one or two plasmid substrates. PUC18 DNA and pBR322 DNA were incubated together with TopoNM. A decrease in the mobility of DNA indicated that the enzyme catalyses inter molecular strand passage reactions leading to the formation of catenated DNA (Fig. 7A). Earlier, we have shown *M. smegmatis* DNA gyrase is a potent decatenase when compared with *E. coli* DNA gyrase (Manjunatha *et al*., 2002). The decatenation activity of TopoNM was determined using the kinetoplast DNA network as substrate. The unlinking of the plasmid DNA from an interlinked DNA network was monitored by agarose gel electrophoresis. Data presented in Fig. 7B show that TopoNM carries out decatenation of the interlinked molecules. The efficiency of decatenation by TopoNM was comparable to *M. smegmatis* DNA gyrase.

Effect of type II topoisomerase inhibitors on TopoNM

Type II topoisomerases from eubacteria are targets for many classes of antibacterials (Maxwell, 1993; Barnard and Maxwell, 2001; Chatterji *et al*., 2001). Among them, quinolones and coumarins are the well studied potent inhibitors, which target the two different subunits of these enzymes (Gormley *et al*., 1996; Wentzell and Maxwell, 2000). Quinolone class of drugs inhibit the enzyme activity by stabilizing the DNA-enzyme covalent complex. Inhibition of 50% activity (IC_{50}) for *M. smegmatis* DNA gyrase is attained at 3.2 μg ml^{-1} concentration of ciprofloxacin (Guillemin *et al*., 1999; Chatterji *et al*., 2001, U.H. Manjunatha, D.R. Radha and V. Nagaraja, unpublished). However, the inhibition of TopoNM activity was observed only at very high concentrations viz. IC_{50} value of 125 μg ml^{-1} (Fig. 8A). Such high level of drug sensitivity is normally associated with quinolone resistant DNA gyrase having mutations in the quinolone resistance determining region (QRDR) (see *Discussion*). Coumarin class of drugs prevent binding of ATP to the enzyme and are competitive inhibitors of enzyme activity (Lewis *et al*., 1996; Maxwell, 1997). Novobiocin inhibits TopoNM activity with an IC_{50} value of 75 μg ml^{-1} (Fig. 8B), which is 150-fold higher than required for the inhibition of mycobacterial DNA gyrase (Chatterji *et al*., 2001). The divergence of TopoN sequence from a typical GyrB sequence with respect to ATP and coumarin binding appears to be the primary reason for the observed resistance of the enzyme to coumarins (see *Discussion*).

Discussion

The functional characterization of the four topoisomerases in *E. coli* provided a general framework on how these enzymes function and share their intracellular work load to solve topological problems in prokaryotes (Wang, 1991; Luttinger, 1995; Espeli *et al*., 2003a; Espeli and Marians, 2004). Previous studies established a role for topoisomerase I in removal of negative supercoils behind transcription bubble, whereas gyrase was shown to exert its effect ahead of these machineries in removing positive supercoils (Liu and Wang, 1987; Champoux, 2001). It was also established that topoisomerase IV carries out relaxation of negatively and positively supercoiled DNA while DNA gyrase takes care of negative supercoiling. In addition, the division of labour between DNA gyrase and topoisomerase IV was unambiguously demarcated in accordance with their respective activities, which established topoisomerase IV as the major decatenase in the

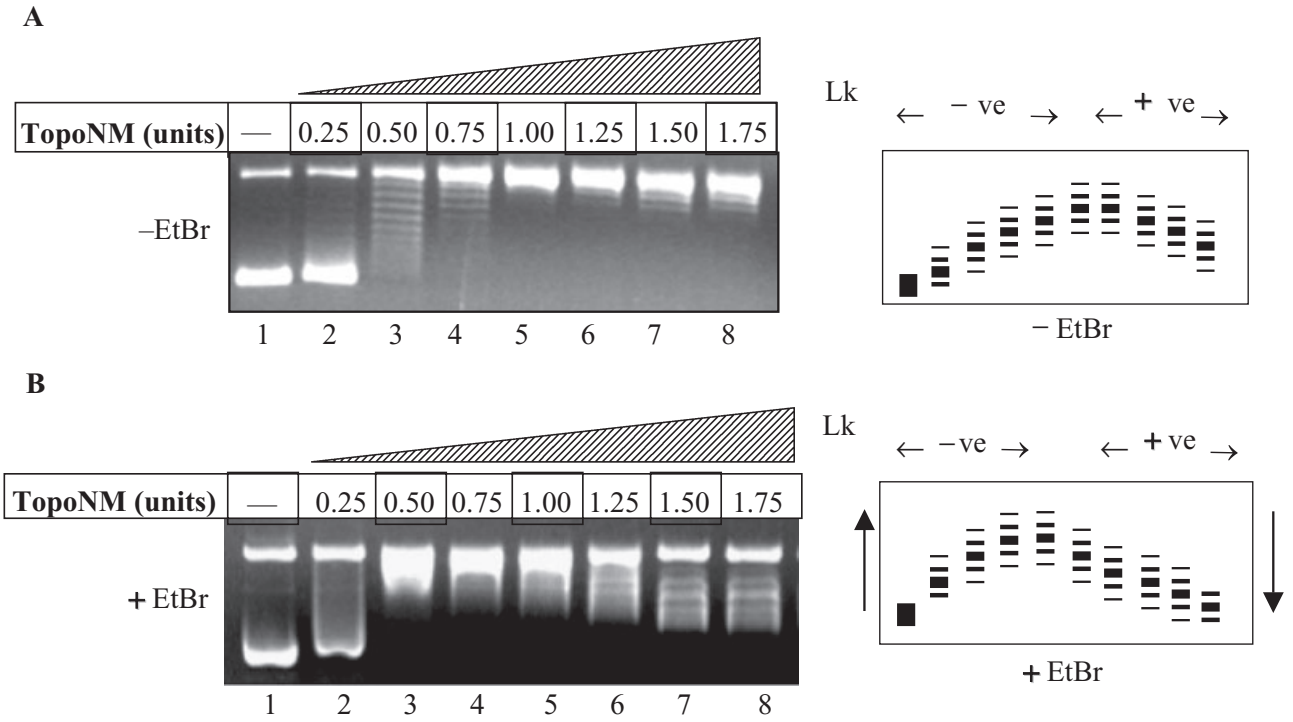

Fig. 4. Positive supercoiling activity of TopoNM.
A. Activity assays were performed using 500 ng of negatively supercoiled pUC18 DNA and increasing concentrations of TopoNM. Reactions were resolved on a 1.2% agarose gel in the absence of EtBr.
B. Identical samples (as shown in A) were also resolved on a 1.2% agarose gel in the presence of 50 ng ml^{-1} EtBr. Lane 1: pUC18 DNA substrate. Lanes 2–8: DNA with 0.25, 0.5, 0.75, 1.0, 1.25, 1.5 and 1.75 units of TopoNM.

cell (Hiasa and Marians, 1996; Espeli *et al*., 2003b; Nurse *et al*., 2003). Furthermore, it was shown that DNA gyrase is the primary target for quinolone class of drugs in Gram-negative organisms, while in Gram-positives, topoisomerase IV is the primary target (Higgins *et al*., 2003). In contrast to the latter typical type II enzymes, TopoNM exhibits positive supercoiling activity, not seen so far with other eubacterial topoisomerases. Among all topoisomerases only reverse gyrases, found in hyperthermophilic eubacteria and archaea which belong to type IA class are shown to introduce positive supercoils into the genome (Forterre *et al*., 2000; Forterre, 2002). The phylogenetic divergence of TopoNM from DNA gyrase or topoisomerase IV clusters also argues against its grouping with topoisomerase IV or DNA gyrase. In contrast to these typical enzymes, TopoNM is highly resistant to quinolone and coumarin class of compounds; conversion of supercoiled pUC18 DNA into relaxed covalently closed circular form is inhibited at drug concentrations that are physiologically irrelevant. Mutations in the QRDR region in the N-terminal region of GyrA are the primary cause for reduced susceptibility of DNA gyrase towards fluoroquinolones. Accordingly a single or double mutations in the QRDR are associated with 16- or 32-fold increase in MICs of the drug resistant enzyme (Kocagoz *et al*., 1996). An Asp to Gly change at position 94 of *M. tuberculosis* GyrA confers ciprofloxacin resistance (Takiff *et al*., 1994). TopoM naturally contains Gly at this position. Similarly, one of the reasons for the observed resistance to novobiocin could be because of the significant variation in the ATPase domain of TopoNM from that of canonical ATP binding pocket found in other topoisomerases. Based on the structure of novobiocin complexed with the GyrB N-terminal domain, sequence alignment and mutation studies with topoisomerase IV, several residues involved in binding of novobiocin to GyrB and ParE have been identified (Lewis *et al*., 1996; Hardy and Cozzarelli, 2003; Bellon *et al*., 2004). In an alignment between TopoN, ParE and GyrB, Tyr109 of GyrB, which is shown to contact novobiocin, is replaced by Asp in TopoN. Gly64, Ile78 and Thr165 of *E. coli* GyrB, when mutated, confer resistance to novobiocin and these residues are naturally replaced by Ser, Lys and Asn, respectively, in TopoN. Moreover, the ATPase domain of TopoN forms an independent branch and does not fall into any of the three clusters formed by GyrB/ParE, topoisomerase II and topoisomerase VI (Fig. 1C). The TopoNM falls into a group different from gyrase and topoisomerase IV even when the ATPase domain of TopoN is not included in the phylogenetic analysis (not shown). Furthermore, the *topoN* and *topoM*

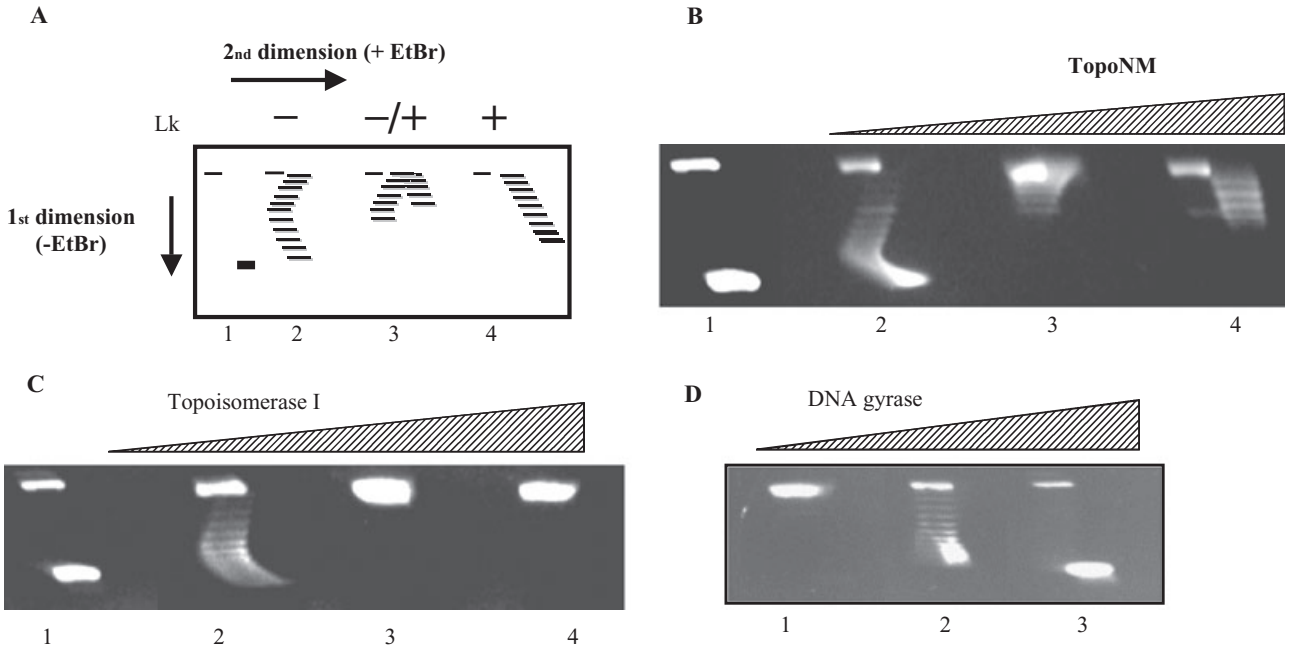

Fig. 5. Topological reactions catalysed by topoisomerases from *M. smegmatis*.
A. Schematic representation of two-dimensional agarose gel electrophoresis showing the migration of positive and negative topoisomers.
B. Reactions with TopoNM. Lane 1: migration of negatively supercoiled pUC18 DNA substrate. Lane 2: partial relaxation of negatively supercoiled DNA at 0.5 units of TopoNM. Lane 3: transition from negatively supercoiled to positively supercoiled DNA at 1.5 units of TopoNM. Lane 4: formation of positively supercoiled DNA at 2.5 units of TopoNM.
C. Reactions with topoisomerase I. Lane 1: negatively supercoiled pUC18 DNA substrate. Lane 2: partial relaxation of negatively supercoiled DNA at 3 units of topoisomerase I. Lane 3: Complete relaxation of the DNA at 6 units of topoisomerase I. Lane 4: no introduction of positive supercoils in DNA at 15 units topoisomerase I.
D. Reaction with DNA gyrase. Lane 1: relaxed pUC18 DNA as substrate. Lane 2: introduction of negative supercoils at 0.5 units of DNA gyrase. Lane 3: complete negative supercoiling of DNA at 1.5 units of DNA gyrase.

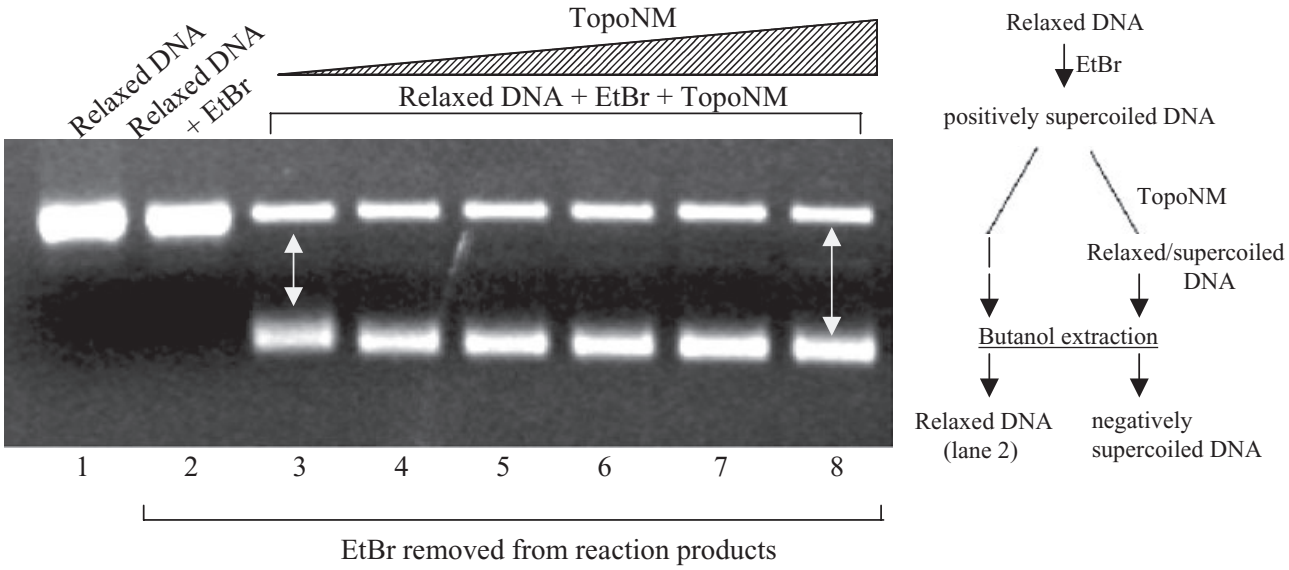

Fig. 6. Activity of TopoNM on positively supercoiled DNA substrate. Relaxed DNA was incubated with 0.2 μg of EtBr to covert it into positively supercoiled DNA and incubated with increasing concentrations of TopoNM. EtBr was removed from the reaction products by butanol extraction, from lanes 2–8, before resolving on 1.2% agarose gel. Lane 1: relaxed DNA. Lane 2: relaxed DNA with EtBr (positively supercoiled DNA) followed by butanol extraction. Lanes 3–8: relaxed DNA with EtBr and 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 units of TopoNM followed by butanol extraction. The arrows between the DNA bands indicate increase in negative supercoiling of DNA with increasing enzyme concentrations. Reaction scheme is shown on right side of the figure.

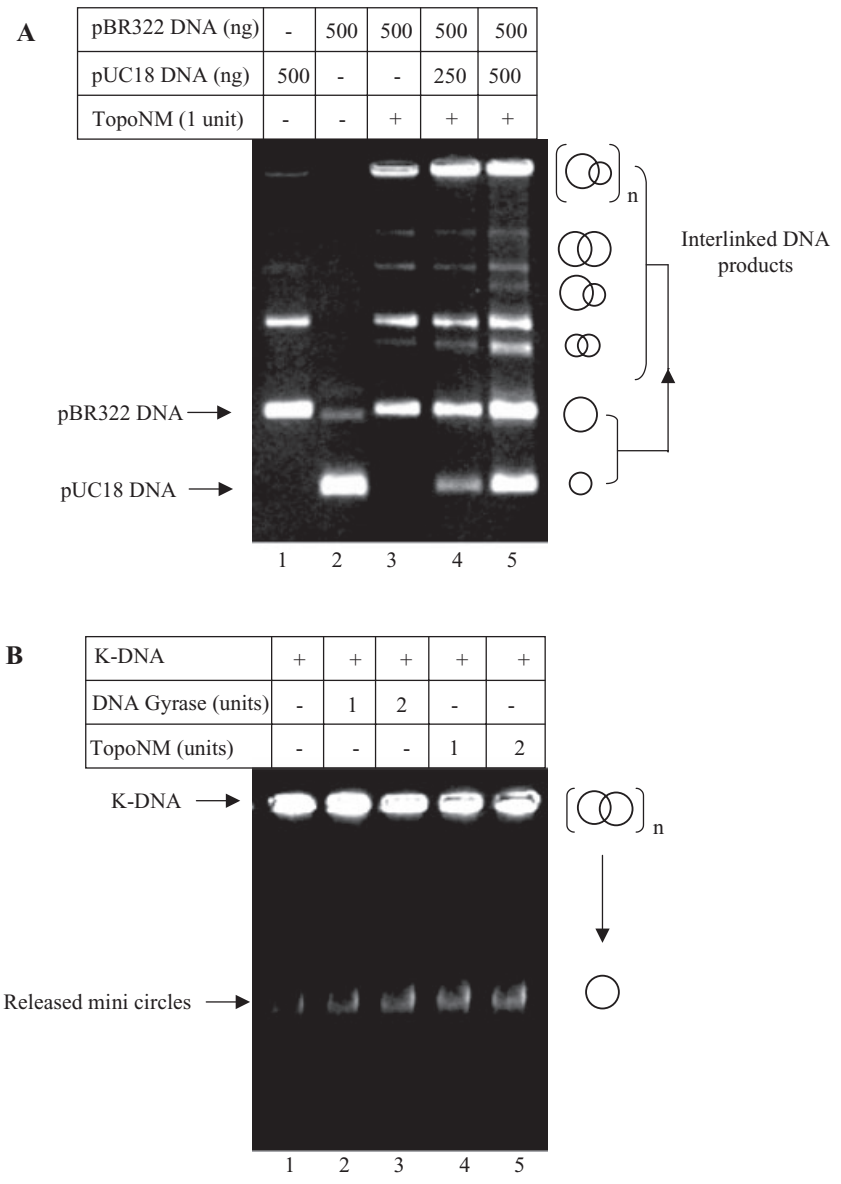

Fig. 7. Catenation and decatenation activity of TopoNM.
A. Catenation assays were carried out using pUC18 and pBR322 DNA and 1 unit of TopoNM. Lane 1, 500 ng of pBR322 DNA. Lane 2, 500 ng of pUC18 DNA. Lane 3, 500 ng of pBR322 DNA with TopoNM. Lane 4, 500 ng of pBR322 DNA and 250 ng of pUC18 DNA with TopoNM. Lane 5, 500 ng of pBR322 DNA and 500 ng of pUC18 DNA with TopoNM.
B. Decatenation assays were carried out with TopoNM and DNA gyrase. Lane 1, Catenated DNA substrate. Lanes 2 and 3, substrate with 1 and 2 units of DNA gyrase respectively. Lanes 4 and 5, substrate with 1 and 2 units of TopoNM respectively.

could not complement *E. coli gyr* and *par ts* strains and also *Salmonella typhimurium parE* and *parC ts* strains (not shown). All these data, together, indicate early divergence of TopoNM genes from rest of the genes that encode type II topoisomerases in eubacteria. Elucidation of a number of topoisomerase II structure have led to a greater understanding of their architecture, enzyme action and reaction mechanisms (Corbett and Berger, 2003, 2004). As TopoNM is overexpressed in large amounts in soluble form, it is amenable for structural studies which would provide clues on its function.

With a number of bacterial genomes deciphered, it has become apparent that many, including *M. tuberculosis*, possess only two topoisomerases viz. DNA topoisomerase I and DNA gyrase. In such a scenario, DNA gyrase would relax the positive torsional stress generated during transcription and replication and also catalyse the decatenation of replicated DNA, taking over the role of topoisomerase IV. In accordance with this, we have shown that mycobacterial DNA gyrase catalyses the decatenation of interlinked molecules efficiently (Manjunatha *et al.*, 2002). Meanwhile, topoisomerase I would remove negative supercoils arising during various DNA transaction processes and prevent excessive negative supercoiling by DNA gyrase. Identification of an additional type II topoisomerase from *M. smegmatis* with unusual properties adds a new twist on the diversity and varied topoisomerase distribution and their intracellular functions in bacterial kingdom. None of the other mycobacterial genomes encode the genes for the new topoisomerase.

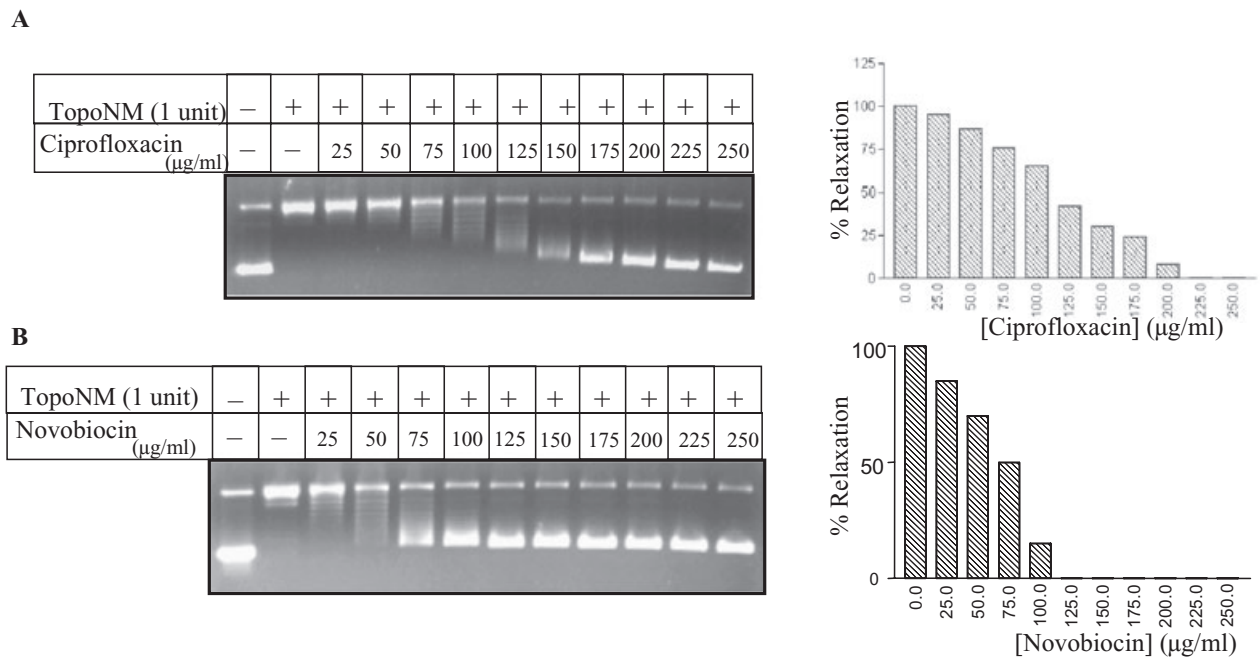

Fig. 8. TopoNM susceptibility to topoisomerase inhibitors. Relaxation assays were performed with 500 ng of negatively supercoiled pUC18 DNA and 1 unit of TopoNM in the presence (A) ciprofloxacin and (B) novobiocin. % relaxation activity is plotted as a function of drug concentration.

M. smegmatis has the largest genome (6.9 Mb) with 6901 genes in comparison to other sequenced mycobacteria (*Mycobacterium avium* 4.83 Mb; *Mycobacterium bovis* 4.35 Mb; *Mycobacterium tuberculosis* CDC1551 4.4 Mb; *M. tuberculosis* H37Rv 4.4 Mb) with number of genes ranging from 3920 to 4350. In all these smaller genomes only topoisomerase I and DNA gyrase manage to accomplish all the topological reactions needed for cell function. Higher organisms with larger genomes have additional topoisomerases to cope up with cellular requirements. Thus, there appears to be some correlation relating genome size and the existence of additional topoisomerase within genus mycobacteria. However, comparison with other eubacterial genomes such as *E. coli* and many others do not reveal such correlation. The latter genomes have an average genome size of 4 Mb and encode full complement of four topoisomerases.

The strong decatenase activity of TopoNM would imply a role for the enzyme in chromosome segregation either playing a back up function to gyrase or an up front role. *M. smegmatis* has 10 times faster growth rates than *M. tuberculosis* and many other mycobacteria and hence additional relaxation and decatenation activities may reflect the requirements of important cellular physiology. The positive supercoiling activity on the other hand may contribute in chromosome compaction. The positive supercoiling activity of reverse gyrases encoded by hyperthermophilic organisms have a role in protecting genome integrity. The DNA chaperone activity of these enzymes impart resistance to genome against high temperature and UV irradiation (Kampmann and Stock, 2004; Napoli *et al.*, 2004). Although the positive supercoiling activity of TopoNM is not comparable with that of reverse gyrases, another possible function could be its role in protecting the genome from a hostile growth environment. The distinctive nature of the enzyme may also represent yet unknown roles in other DNA transaction processes.

Experimental procedures

Computational analysis

Mycobacterium smegmatis genome sequence was analysed using WU-BLAST (http://tigrblast.tigr.org/cmr-blast). Presence of various domains in the proteins were predicted using pfam algorithm. Proteins annotated as GyrB and ParE were aligned with TopoN; and those annotated as GyrA and ParC were aligned with TopoM using CLUSTALW. MEGA version 2 was used for phylogenetic analysis. The trees were constructed using the neighbour-joining method (Kumar *et al.*, 2001).

Plasmid construction

To construct pRSET-*topoN* (pJPN-N) and pRSET-*topoM* (pJPN-M), the ORFs corresponding to *topoN (*2094 nucleotides) and *topoM (*2139 nucleotides) were amplified from *M. smegmatis* MC2 chromosomal DNA by polymerase chain reaction (PCR) (5′-CCATCGATAAGATCTCATATGTCTGAT CCGGGCGTCTACCA-3′ and 5′-GCTCTAGACCTAAGTCAG

GTCCAGCGCG-3′ or 5′-CCATCGATAAGATCTCATATGAC CGCCACCTTGGACGTTCC-3′ and 5′-GCTCTAGACCTAC TCCGCGGGGGCGGT-3′ respectively). The PCR fragments were digested with NdeI and XbaI and cloned into unique NdeI and XbaI sites of pRSET-A (Invitrogen).

Protein expression and purification

BL26(DE3) cells harbouring either pJPN-N or pJPN-M were grown till 0.6 OD_{600} and induced with 0.3 mM IPTG for 3 h at 37°C. Cell free protein extracts were prepared in buffer A (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 50 mM KCl, 5 mM β-mercaptoethanol and 5% glycerol) and centrifuged at 100 000 *g* for 2 h. After 0–65% ammonium sulphate fractionation of the supernatant, the pellet fraction was dialysed against buffer B (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM β-mercaptoethanol and 5% glycerol). Both TopoN and TopoM were purified using Hitrap-heparin followed by Hitrap-mono Q column chromatography with a linear gradient of 0.05–1.0 M NaCl. The protein samples were resolved on an 8% glycine-acrylamide SDS-PAGE (Laemmli, 1970). Gels were stained with Coomassie blue R-250 (0.1% in 10% acetic acid, 10% methanol) and then destained with 10% methanol, 10% acetic acid. The active protein fractions were dialysed against buffer C (50 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 1 mM β-mercaptoethanol and 10% glycerol) and stored at –70°C. Unit activity for TopoNM was defined as the amount of enzyme required for complete relaxation of 500 ng of negatively supercoiled pUC18 DNA in 30 min at 37°C.

Mycobacterium smegmatis topoisomerase I and DNA gyrase were purified as described previously (Bhaduri *et al*., 1998; Manjunatha *et al*., 2002).

Relaxation of plasmids

Negatively supercoiled pUC18 plasmid was incubated with *M. smegmatis* topoisomerase I in a reaction buffer containing 40 mM Tris-HCl, pH 8.0, 20 mM KCl, 5 mM $MgCl_2$ at 37°C for 45 min. Reactions were heat inactivated at 65°C for 30 min. DNA was recovered by ethanol precipitation.

Topoisomerase assays

Relaxation and supercoiling assays with TopoNM and DNA gyrase were carried out in buffer containing 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM DTT, 1 mM spermidine-HCl, 20 mM KCl, 1 mM ATP, 50 μg ml^{-1} bovine serum albumin and 500 ng of negatively supercoiled or relaxed pUC18 DNA at 37°C for 30 min. Reactions were terminated by the addition of 50 mM EDTA, 1% SDS and 50 μg ml^{-1} proteinase K (Peng and Marians, 1993; Manjunatha *et al*., 2002). Relaxation assays with topoisomerase I were carried out in a buffer containing 40 mM Tris-HCl, pH 8.0, 20 mM NaCl, 5 mM $MgCl_2$ and 500 ng of negatively supercoiled pUC18 DNA at 37°C for 30 min as described previously (Bhaduri *et al*., 1998).

Catenation assays were carried out in buffer containing 40 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 10 mM DTT, 100 mM potassium glutamate, 1.4 mM ATP, 50 μg ml^{-1} bovine serum albumin, 15% PEG, 500 ng of supercoiled pUC18 DNA (or pUC18 and pBR322 DNA together), were incubated with 2.5 units of TopoNM at 37°C for 30 min.

Decatenation assays were carried out using 500 ng of kinetoplast DNA (K-DNA) in buffer containing 40 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 10 mM DTT, 100 mM potassium glutamate, 40 μM ATP, 50 μg ml^{-1} bovine serum albumin and 500 ng of catenated DNA at 37°C for 30 min. The reaction products were resolved on a 1.2% agarose gel and visualized with EtBr staining.

One dimension gel electrophoresis

DNA topoisomers were resolved in 1.2% agarose gel in the absence of EtBr in TAE buffer (unless indicated). To differentiate the positively supercoiled topoisomers with negatively supercoiled topoisomers in one dimension, gels were resolved in presence of 50 ng ml^{-1} EtBr. Gel electrophoresis was carried out at 5 v cm^{-1} for 16 h.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed in TBE buffer, at 5 v cm^{-1} for 16–17 h, in first dimension and 2 v cm^{-1} for 15 h in second dimension. Relaxed to supercoiled plasmids were fractionated on a 1.2% agarose gel in the absence of EtBr and negative and positively supercoiled plasmids were fractionated in presence of 30 ng ml^{-1} EtBr during the second dimension. Negatively supercoiled pUC18 DNA was used as substrate for topoisomerase I and TopoNM. Relaxed pUC18 DNA was used as substrate for DNA gyrase. Gels were stained with EtBr (1 mg ml^{-1}). Stained gels were photographed under UV light using the Bio-Rad gel documentation system.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. TopoN and TopoM were dialysed against buffer C lacking glycerol. A total of 150 μg of TopoN, 50 μg of TopoM and TopoNM (reconstituted with same amounts of TopoN and TopoM) were loaded onto a 25 ml Superdex S-200 column equilibrated with buffer C (lacking glycerol) at a flow rate of 0.5 ml min^{-1}.

A. Absorbance at 214 nm ploted against elution volume.

B. Determination of Stokes radius of proteins eluting in different elution volumes following calibration of the column using thyroglobulin, catalase, albumin, ovalbumin, chemotrypsinogen A as standards.

Fig. S2. Negatively supercoiled pUC18 DNA (0.175 pmol) was incubated with 0.1–1.6 pmol of TopoNM in the relaxation assay buffer lacking ATP (40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM DTT, 1 mM spermidine-HCl, 20 mM KCl and 50 μg ml^{-1} bovine serum albumin). After 10 min 0.1 pmol of *M. smegmatis* topoisomerase I was added and the reactions were incubated for 2 h at 37°C. The reactions were terminated by the addition of 0.1% SDS and were treated with proteinase K to a final concentration of 50 μg ml^{-1} for 30 min at 37°C. The reaction products were resolved on a 0.1% agarose gel in the absence of EtBr and the topoisomers were visualized after staining with EtBr.

Fig. S3. Relaxed pUC18 DNA (500 ng) was incubated with 2 μg ml^{-1} of EtBr in the relaxation assay buffer of TopoNM lacking ATP to generate positively supercoiled DNA. The positively supercoiled DNA was incubated with TopoNM either in presence or absence of 1 mM ATP at 37°C for 30 min. After the reaction, EtBr was removed by butanol extraction and the products were resolved by agarose gel electrophoresis. Lane 1: relaxed pUC18 DNA; lane 2: positively supercoiled DNA after butanol extraction. Lanes 3 and 4: positively supercoiled DNA with 1 and 2 units of TopoNM in the presence of ATP. Lanes 5 and 6: positively supercoiled DNA with 1 and 2 units of TopoNM in the absence of ATP.

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