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Functional characterisation of mycobacterial DNA gyrase: an efficient decatenase

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ABSTRACT

A rapid single step immunoaffinity purification procedure is described for *Mycobacterium smegmatis* DNA gyrase. The mycobacterial enzyme is a 340 kDa heterotetrameric protein comprising two subunits each of GyrA and GyrB, exhibiting subtle differences and similarities to the well-characterised *Escherichia coli* gyrase. In contrast to *E.coli* gyrase, the *M.smegmatis* enzyme exhibits strong decatenase activity at physiological Mg^{2+} concentrations. Further, the enzymes exhibited marked differences in ATPase activity, DNA binding characteristics and susceptibility to fluoroquinolones. The holoenzyme showed very low intrinsic ATPase activity and was stimulated 20-fold in the presence of DNA. The DNA-stimulated ATPase kinetics revealed apparent $K_{0.5}$ and k_{cat} of 0.68 mM and 0.39 s^{-1}, respectively. The dissociation constant for DNA was found to be 9.2 nM, which is 20 times weaker than that of *E.coli* DNA gyrase. The differences between the enzymes were further substantiated as they exhibited varied sensitivity to moxifloxacin and ciprofloxacin. In spite of these differences, mycobacterial DNA gyrase is a functionally and mechanistically conserved enzyme and the variations in activity seem to reflect functional optimisation for its physiological role during mycobacterial genome replication.

INTRODUCTION

DNA topoisomerases are a ubiquitous class of enzymes that catalyse the interconversions of various topological forms of DNA. Topological transformations are achieved by passing either one strand of DNA through another single strand or passing a duplex DNA through another transient double-stranded cleavage held by the enzyme, followed by religation of the cleaved strands. DNA gyrase belongs to the type II class of topoisomerases, catalysing DNA supercoiling/relaxation, catenation/decatenation, knotting/unknotting, etc. (1–3). Although enzymes belonging to class II are structurally and mechanistically related, it appears that they have acquired distinct characteristics during evolution. Among these, DNA gyrase is exclusively present in prokaryotes and is essential for cell survival. Moreover, the ability of DNA gyrase to introduce negative supercoils into DNA is a unique feature amongst all known type II topoisomerases.

Escherichia coli DNA gyrase is composed of two subunits, A and B. The active gyrase is a heterotetramer (A_2B_2). The GyrA N-terminal domain contains the site for DNA breakage and reunion while the C-terminal domain has a DNA wrapping function (1). The GyrB N-terminal domain has an ATPase function whereas the C-terminal domain interacts with the GyrA subunit and has also been found to play a role in DNA binding (4,5).

The *gyrA* and *gyrB* genes have been characterised from a variety of bacterial species, primarily with the aim of determining the molecular basis of drug resistance. Sequence analysis reveals that important motifs in DNA gyrase from different bacteria are highly conserved (6,7). In spite of the conservation of motifs involved in catalysis, eubacterial DNA gyrases appear to fall into two subclasses based on sequence alignment and other properties (5,8,9). A stretch of 163–168 amino acids present in the C-terminal domain of GyrB of gram-negative bacteria is absent in GyrB of gram-positive bacteria. The differences in DNA gyrases from the two subclasses are further apparent from studies on antibody cross-reactivity (8) and differential quinolone susceptibility (10,11). These differences may reflect subtle alterations in the biochemical properties of the enzymes from two subclasses, influencing enzyme function. The analysis of DNA gyrase from mycobacteria, which are gram-positive acid-fast bacilli, is also important from a different perspective. *Mycobacterium tuberculosis*, *Mycobacterium leprae* and many other species belonging to this genus form one of the most formidable groups of pathogenic bacteria and elucidating the properties of DNA gyrase would be necessary to develop it as a drug target. With the emergence of multidrug-resistant strains of *M.tuberculosis* it has also become necessary to study enzymatic pathways essential for cell survival.

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Monoclonal antibodies against the individual subunits of mycobacterial DNA gyrase have been characterised (9,12). Among the panel of mAbs, MsGyrB:A4, a GyrB-specific mAb, co-immunoprecipitates GyrA along with the GyrB subunit (12). This has aided facile immunoaffinity purification and the biochemical characterisation of *Mycobacterium smegmatis* DNA gyrase to reveal the important features of its activity.

MATERIALS AND METHODS

Bacterial strains and materials

Mycobacterium smegmatis SN2 was obtained from our laboratory collection. *Mycobacterium smegmatis* mc^2155 and its ciprofloxacin-resistant strain were obtained from P. K. Chakraborti (Institute of Microbial Technology, Chandigarh, India). *Mycobacterium smegmatis* was grown in modified Youman and Karlson's medium with 0.2% Tween-80 at 37°C (13). The resistant strain was grown in the presence of ciprofloxacin (32 $\mu g\ ml^{-1}$). The enzyme-conjugated anti-mouse and anti-rabbit secondary antibodies and Enhanced Chemiluminescence Kit were from Amersham Pharmacia Biotech (Little Chalfont, UK). *Escherichia coli* DNA gyrase overexpression clones were obtained from A. Maxwell (John Innes Centre, UK). *Leishmania donovani* kinetoplast DNA was a kind gift from H. K. Majumder (Indian Institute of Chemical Biology, India). Dimethyl pimelimidate and ciprofloxacin hydrochloride (ciprofloxacin) were from Sigma. Moxifloxacin hydrochloride (moxifloxacin) was a gift from H. Dornauer and S. K. Arora (Lupin Ltd, India).

Anti-GyrB immunoaffinity purification of DNA gyrase

Monoclonal antibody MsGyrB:A4 was purified from ascitic fluid using protein A–Sepharose affinity column chromatography. Purified mAb MsGyrB:A4 was covalently linked to protein A–Sepharose (7 ml) using 20 mM dimethyl pimelimidate as described (14). The coupled matrix was regenerated with 10 mM glyine–HCl, pH 2.8, and equilibrated with TEM buffer [35 mM Tris, pH 7.4, 5 mM EDTA, 2 mM β-mercaptoethanol (βME)]. The *M.smegmatis* cells (12 g) were sonicated in TEM buffer containing 10% glycerol and the homogenate was centrifuged at 12 000 r.p.m. for 15 min. The crude cell lysate was then subjected to ultracentrifugation at 40 000 r.p.m. for 2 h followed by 30–55% ammonium sulfate fractionation of the supernatant. The ammonium sulfate pellet was dissolved in buffer A (TEM buffer containing 50 mM NaCl) and dialysed against the same buffer. Binding of the dialysed protein to antibody-coupled beads (7 ml) was carried out in buffer B (buffer A with 1% Nonidet P-40). The column was washed with 10 column vol of buffer B followed by 10 column vol of buffer A. GyrA subunit was eluted with buffer C (TEM with 500 mM NaCl) followed by elution of GyrB in buffer D (0.1 M glycine–HCl, 500 mM NaCl, pH 2.8). The GyrB fractions were immediately neutralised with Tris–HCl, pH 9.0. The peak fractions of subunits A and B were pooled separately, dialysed against potassium glutamate buffer (35 mM Tris–HCl, 100 mM potassium glutamate, 2 mM βME and 10% glycerol) and frozen in liquid nitrogen and stored at –70°C. DNA gyrase from *M.smegmatis* mc^2155 and its ciprofloxacin-resistant strain were purified as described above.

SDS–PAGE and western blot analysis

Proteins were resolved on an 8% SDS–PAGE gel followed by silver staining (15) of the gel or transfer to polyvinylidine difluoride membrane for immunodetection of the GyrA and GyrB subunits as described (9).

Gel filtration chromatography

DNA gyrase (200 U) was applied to a Superdex-200 column (10 × 30 HR; Amersham Pharmacia Biotech), equilibrated with potassium glutamate buffer at a flow rate of 0.2 $ml\ min^{-1}$ using an Akta FPLC system (Amersham Pharmacia Biotech) and fractions (0.4 ml each) were collected. The molecular weights of the proteins eluting in different fractions were estimated following calibration of the column using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and cytochrome c (12.3 kDa) as standards.

DNA supercoiling and relaxation assays

Relaxed DNA was prepared by treating supercoiled pUC18 DNA with purified *E.coli* topoisomerase I (16). *Escherichia coli* DNA gyrase was purified as described by Maxwell and Howells (17). The supercoiling reactions were carried out as described earlier (9). One unit of gyrase was defined as the concentration of enzyme that catalyses the conversion of 400 ng relaxed pUC18 DNA into the completely supercoiled form in 1 h at 37°C. The IC_{50} was defined as the drug concentration that reduced the enzymatic activity observed with drug-free controls by 50%. Relaxation assays were carried out with 10 U enzyme in the supercoiling buffer devoid of ATP using supercoiled DNA as substrate for 6 h at 37°C.

Decatenation reaction

The assays were carried out under supercoiling reaction conditions using 500 ng kinetoplast DNA. The products of the reaction were quantitated using a Bio-Rad gel documentation system. To measure the effect of Mg^{2+} on decatenation and supercoiling activities, a range of $MgCl_2$ concentrations was used in the reaction buffer. The decatenation activities of the *E.coli* and mycobacterial DNA gyrases were normalised with respect to supercoiling units.

ATPase assay

The ATPase reactions (30 µl) were carried out in supercoiling buffer containing 0.05–4 mM ATP, 10 $\mu g\ ml^{-1}$ DNA (240 bp from pBR322) and 0.02 µCi [α-^{32}P]ATP (3000 Ci $mmol^{-1}$). For intrinsic ATPase activity, 450 nM individual subunits, and for ATPase kinetics 75 nM gyrase were used. Reactions were carried out for 30 min at 37°C and terminated by the addition of an equal volume of chloroform. The aqueous layer (1 µl) was resolved by polyethyleneimine–cellulose thin layer chromatography with 1.2 M $LiCl_2$ and 0.1 mM EDTA. The spots corresponding to ATP and ADP were quantitated using a phosphorimager (Fuji Film FLA 2000).

Electrophoretic mobility shift assay (EMSA)

Assays were carried out using a PCR-amplified 240 bp DNA fragment encompassing the strong gyrase site (SGS) from pBR322 (18) using specific primers (forward primer, 5′-CAA GCC GTC GAC ACT GGT CCC GCC A-3′; reverse primer,

5′-CGC GAG GGA TCC TTG AAG CTG-3′). The amplified fragment of DNA was restricted with *Bam*HI and end-filled using [α-^{32}P]ATP (3000 Ci mmol^{-1}) and Klenow fragment of DNA polymerase I. The labelled DNA (0.1 × 10^{-9} M) was incubated with varying concentrations of enzyme in supercoiling buffer for 30 min at 4°C. The samples were electrophoresed in a 3.5% native polyacrylamide gel using 0.5× TBE buffer containing 10 mM $MgCl_2$ at 4°C. Free and bound complexes were quantitated using a phosphorimager.

Cleavage assay and cleavage site mapping

pBR322 (5 µg) was linearised with *Eco*RI and used to analyse the cleavage pattern of *E.coli* and *M.smegmatis* gyrases. An aliquot of 500 ng DNA was incubated in supercoiling buffer along with 75 nM *E.coli* or *M.smegmatis* DNA gyrase in the presence of 1.2 or 30 µg ml^{-1} ciprofloxacin, respectively, for 10 min at 37°C. The DNA–gyrase–quinolone complex was trapped by addition of SDS (0.16%) followed by proteinase K (90 µg ml^{-1}) digestion for 30 min at 37°C. The samples were heat denatured at 90°C for 5 min and analysed by 1% agarose gel electrophoresis.

A PCR-amplified 240 bp DNA fragment was used as the substrate for DNA cleavage reactions. The top or bottom strands of the DNA fragments were labelled using 5′-labelled forward or reverse primers. The cleavage reaction (12.5 µl) was carried out in supercoiling buffer with labelled DNA fragment (0.1 × 10^{-9} M) and 25 nM mycobacterial gyrase in the presence of 30 µg ml^{-1} ciprofloxacin for 10 min at 37°C. The DNA–gyrase–quinolone complex was trapped by addition of SDS (0.16%) followed by proteinase K (90 µg ml^{-1}) digestion for 30 min at 37°C. The DNA was purified by phenol/chloroform extraction and ethanol precipitation and then resuspended in water. Loading dye (95% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 10 mM EDTA) was added to the samples, which were heat denatured at 90°C for 4 min and chilled on ice. The samples were applied to a 6% denaturing polyacrylamide gel. Dideoxy sequencing reactions were carried out using the respective labelled primer.

RESULTS

Immunoaffinity purification of DNA gyrase

We have taken advantage of the high affinity interaction of GyrB with a monoclonal antibody and the physical association of the two subunits of DNA gyrase for immunoaffinity purification of the *M.smegmatis* enzyme. The flowchart for the facile purification procedure is presented in Figure 1A and described in Materials and Methods. We have previously shown that the GyrA–GyrB interaction is predominantly ionic in nature (12). This property was conveniently used to elute GyrA from a GyrB-specific column while bound GyrB was recovered using conditions that disrupt antigen–antibody interactions (Fig. 1B and C). The apparent molecular weights of the GyrA and GyrB polypeptides were 93 and 78 kDa, respectively (Fig. 1B), which correspond to the expected molecular masses deduced from the primary amino acid sequence (19). The present method is rapid, yielding apparently homogeneous individual subunits in a single step. Moreover, unlike the novobiocin–Sepharose affinity chromatography method (20), the elution

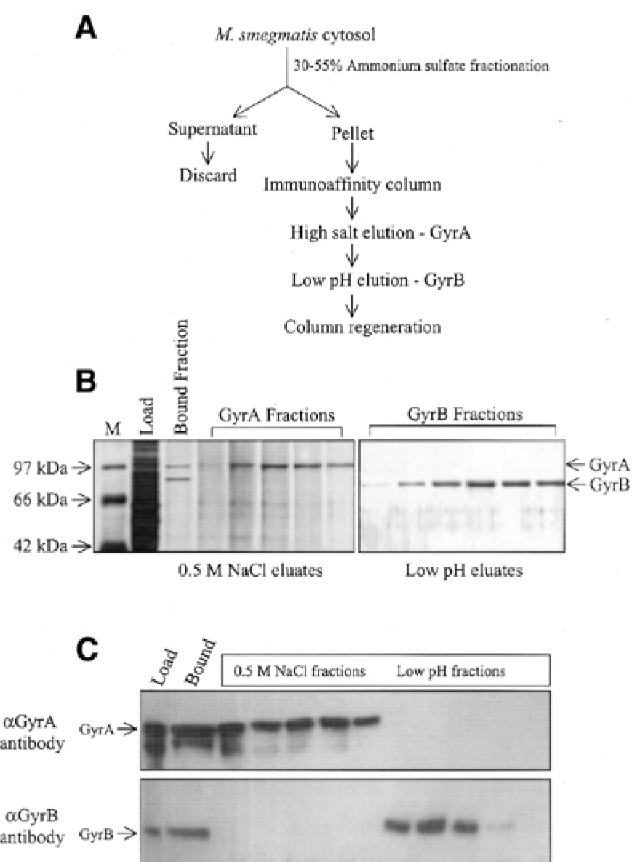

Figure 1. Immunoaffinity purification of *M.smegmatis* DNA gyrase. (**A**) Schematic representation of the purification protocol detailed in Materials and Methods. (**B**) SDS–PAGE and silver staining of the affinity-purified fractions. M represents size markers with the indicated molecular masses. (**C**) Western blot analysis of eluted fractions probed with GyrA- and GyrB-specific antibodies. Load and bound represent the protein loaded on and bound to the column, respectively.

conditions are milder and devoid of elaborate renaturation steps.

Functional characterisation of the affinity-purified DNA gyrase complex

The enzymatic activities of the holoenzyme and the individual subunits were determined as described in Materials and Methods. When individual fractions containing the GyrA and GyrB subunits were assayed separately, no supercoiling activity was observed, indicating the absence of contamination of one subunit with another. Reconstitution of holoenzyme from the subunits resulted in the supercoiling of relaxed pUC18 DNA (not shown). To determine the quaternary structure and active form of gyrase, the holoenzyme was subjected to gel filtration analysis. The active peak corresponds to an apparent mass of 340 kDa. The presence of both the subunits in equal proportions in the active fractions was confirmed by western blot analysis. The specific activity of the enzyme was determined by titrating the individual subunits against each other and was found to be 5.4 × 10^4 and 3.3 × 10^4 U mg^{-1} for the GyrA and GyrB proteins, respectively. One unit of gyrase comprised 10 nM GyrA and 20 nM GyrB subunits. Although the enzyme is a heterotetramer with equal molar

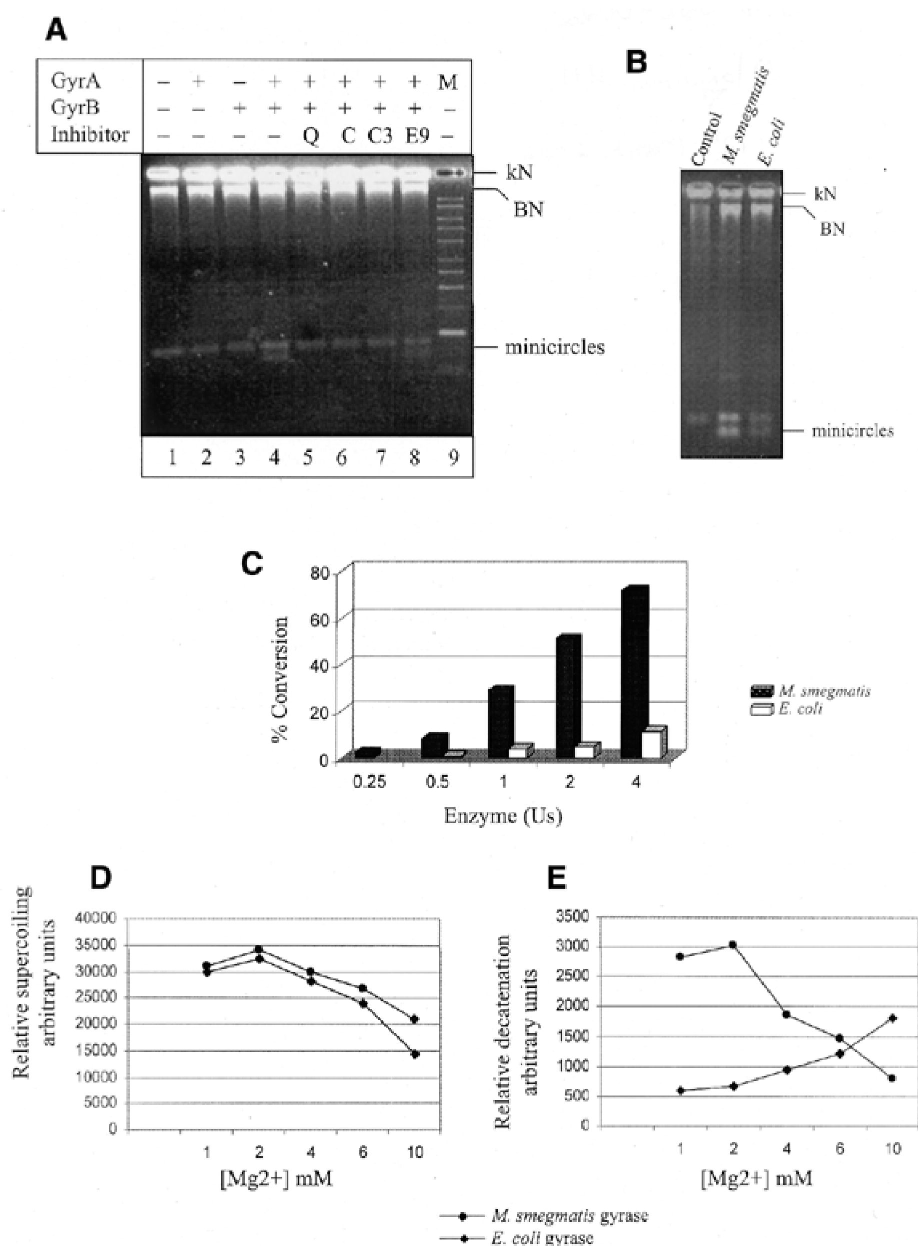

Figure 2. Characteristics of DNA gyrase decatenation activity. (**A**) *Mycobacterium smegmatis* holoenzyme is required for decatenation activity. The reaction, containing 0.5 µg kinetoplast DNA with individual subunits alone (lanes 2 and 3) or together (lanes 4–8), was carried out in the presence of 10 µg ml^{-1} ciprofloxacin (lane 5), 20 µg ml^{-1} novobiocin (lane 6) and 20 µg ml^{-1} each of GyrA-specific mAbs C3 (lane 7) and E9 (lane 8). (**B**) The decatenation reaction was carried out with *M.smegmatis* (2 U) and *E.coli* (4 U) DNA gyrases. M, 1 kb DNA ladder; kN, kDNA network; BN, broken network. (**C**) Quantitative analysis of minicircles released with various concentrations of *E.coli* and *M.smegmatis* enzyme. (**D** and **E**) Quantitative analysis of the effect of Mg^{2+} concentration on DNA supercoiling and decatenation activities of *E.coli* and *M.smegmatis* enzymes.

ratios of GyrA and GyrB, more GyrB was required to reconstitute unit activity. This may be due to reduced specific activity of the GyrB subunit. A similar ratio of 1:2 (A:B) for supercoiling activity has been observed earlier for *E.coli* DNA gyrase (21). The specific activity of DNA gyrase holoenzyme was 20 400 U mg^{-1} protein. In addition to ATP, the DNA supercoiling reaction requires $MgCl_2$ at an optimum concentration of 2 mM. Similar to *E.coli* gyrase, the mycobacterial enzyme showed weaker DNA relaxation than supercoiling activity. The ATP-independent DNA relaxation activity of mycobacterial DNA gyrase is observed with 10 U enzyme, which is comparable with that of *E.coli* gyrase. Novobiocin, a coumarin class of gyrase inhibitor, inhibited DNA supercoiling without affecting DNA relaxation activity of the enzyme (not shown).

Mycobacterium smegmatis DNA gyrase is an efficient decatenase

The decatenation of kDNA to minicircles by mycobacterial DNA gyrase requires both the GyrA and GyrB subunits (Fig. 2A, lanes 2–4). In addition, it also requires Mg^{2+} and ATP. Reactions of DNA gyrase that require energy transduction are inhibited by the coumarin class of antibiotics (1,22)

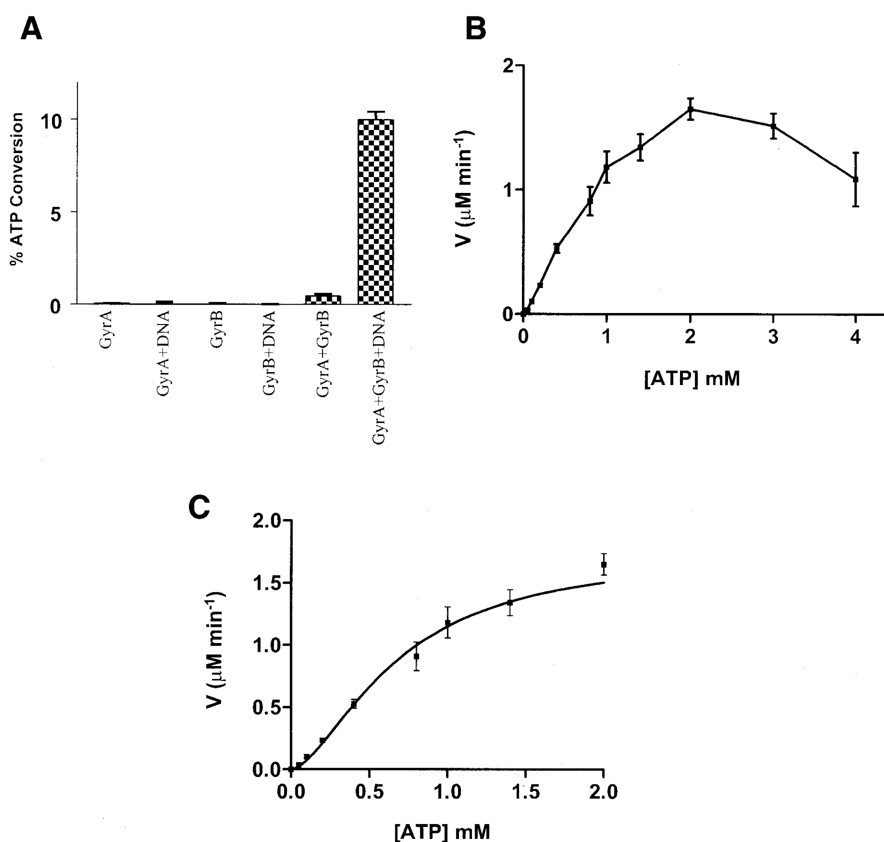

Figure 3. ATPase activity of *M.smegmatis* DNA gyrase. (**A**) Intrinsic and DNA-stimulated ATPase activity of purified individual subunits and holoenzyme. Assays were carried out with 450 nM each of GyrA and GyrB, in the presence of 1.4 mM ATP and 10 µg ml^{-1} linear DNA. (**B**) The rate of DNA-dependent ATP hydrolysis by mycobacterial DNA gyrase (75 nM) at various substrate concentrations ranging from 0.05 to 4 mM ATP, in the presence of 10 µg ml^{-1} 240 bp DNA fragment. (**C**) Non-linear regression analysis of ATP hydrolysis rates with ATP concentrations of 0.05–2.0 mM. All the experiments were repeated three times independently and plotted.

whereas those involving DNA strand breakage and reunion are affected by the quinolone class of drugs (23). The decatenation reaction by mycobacterial enzyme is sensitive to both drugs (Fig. 2A, lanes 5 and 6). Earlier we characterised a GyrA-specific mAb (C3) that inhibited DNA supercoiling activity (9). This mAb inhibited decatenase activity completely, showing that the activity is intrinsic to DNA gyrase (Fig. 2A, lanes 7 and 8).

Escherichia coli DNA gyrase is a relatively poor decatenase when compared with its supercoiling activity (24,25). Decatenation activity of the mycobacterial enzyme was compared with that of *E.coli* by normalising to the supercoiling reaction under identical conditions. The results shown in Figure 2B and C reveal that the mycobacterial enzyme was ~7-fold more efficient in unlinking the kDNA network to minicircles than the *E.coli* enzyme. Unlike *E.coli* DNA gyrase, which requires higher Mg^{2+} concentrations for decatenation activity than DNA supercoiling (Fig. 2D and E), 1–2 mM Mg^{2+} was optimal for both activities of *M.smegmatis* gyrase (Fig. 2D and E). At higher Mg^{2+} concentrations a decrease in decatenation activity was observed (Fig. 2E). Given that the intracellular Mg^{2+} concentration is 1–2 mM (26), the strong decatenase activity of *M.smegmatis* enzyme is of physiological significance.

Kinetics of ATP hydrolysis

To assess the intrinsic ATPase activity of the *M.smegmatis* GyrB subunit and its stimulation in the presence of GyrA and DNA, ATPase assays were carried out. ATPase activity intrinsic to GyrB was not detected even at 450 nM GyrB. However, in the presence of GyrA, a low level of ATP hydrolysis was observed (0.5% conversion of ATP to ADP) (Fig. 3A). Inclusion of both GyrA and DNA resulted in a 20-fold stimulation of ATP hydrolysis. The hydrolysis of ATP is GyrB-specific as inclusion of novobiocin (20 µg ml^{-1}) abolished ATPase activity completely.

The DNA-dependent ATPase kinetics of *M.smegmatis* enzyme were analysed as a function of substrate concentration ranging from 0.05 to 4 mM ATP (Fig. 3B). At substrate concentrations >2 mM, a decrease in the rate of ATP hydrolysis was observed, suggesting substrate inhibition. The reaction product (ADP) has also been reported to be inhibitory to gyrase (27). This may not be of much significance, since the inhibitory ATP concentration may be higher than physiological levels. It has been observed that the kinetics of ATP hydrolysis by *E.coli* DNA gyrase cannot simply be fitted to the Michaelis–Menten equation (28). The deviation has been attributed to the presence of two binding sites for ATP per holoenzyme and the absolute requirement of dimerized functional GyrB subunit (28–30). This seems to be the case with *M.smegmatis* DNA gyrase. The ATP hydrolysis rates at non-inhibitory substrate concentrations (up to 2 mM) were fitted to the Hill equation, $V = V_{max}[S]^h/K^h_{0.5} + [S]^h$, using Graphpad-prism (Fig. 3C). The derived apparent $K_{0.5}$ and V_{max}

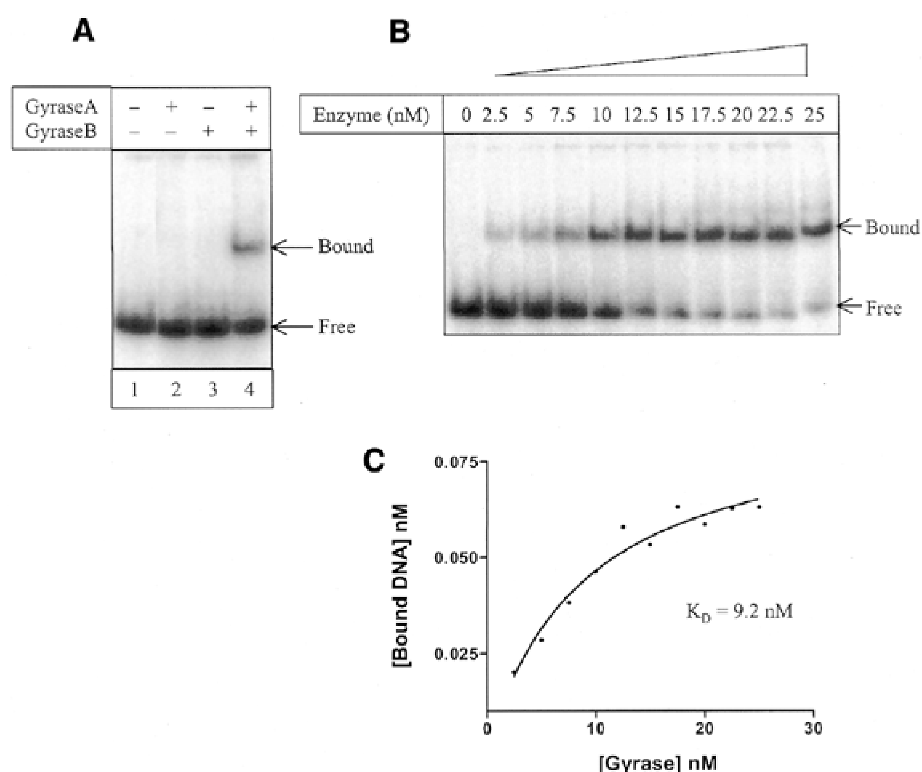

Figure 4. DNA binding analysis of *M.smegmatis* DNA gyrase. EMSAs using the 240 bp labelled DNA fragment from pBR322. (**A**) Assay with individual subunits alone (lanes 2 and 3) or holoenzyme (lane 4). (**B**) Labelled DNA was incubated with varying concentrations of enzyme. The free and bound fractions of DNA were separated on a 3.5% native gel at 4°C and analysed by phosphorimager. (**C**) The free and bound fractions were quantitated by phosphorimager. The line drawn is the theoretical curve for the formation of a bimolecular complex between gyrase and DNA. The dissociation constant (K_d) of gyrase–DNA is shown.

were 0.68 mM and 1.76 µmol l^{-1} min^{-1}, respectively, and turnover of the mycobacterial enzyme was found to be 0.39 s^{-1}. These values are lower than the *E.coli* DNA gyrase (see Discussion). The Hill coefficient (number of binding sites) of 1.7 implies a positive cooperativity between the two ATP-binding sites.

DNA binding by *M.smegmatis* DNA gyrase

In EMSAs the individual subunits of DNA gyrase did not bind to DNA, whereas reconstituted holoenzyme showed binding (Fig. 4A). We have previously demonstrated that a 165 amino acid stretch present in the C-terminal domain of *E.coli* GyrB contributes to DNA binding. The reconstitution of *E.coli* GyrA with deletant GyrB did not bind to DNA (5). In contrast, the 165 amino acid stretch is not found in GyrB from mycobacteria and other gram-positive bacteria. The results presented in Figure 4A thus indicate the importance of alternate regions in mycobacterial GyrB for DNA binding. To measure the dissociation constant of the enzyme with DNA, we carried out binding studies with fixed DNA and varying enzyme concentrations (Fig. 4B). The data were directly fitted to the binding isotherm (Fig. 4C) and the dissociation constant (K_d) was estimated to be 9.2 nM. The *E.coli* enzyme exhibits a K_d in the range 0.2–0.5 nM (31,32), values ~20-fold lower than *M.smegmatis* DNA gyrase.

DNA cleavage by mycobacterial DNA gyrase

Escherichia coli DNA gyrase cleaves DNA at preferred sites in the presence of quinolones (18,33). Highly preferred sites are referred to as SGSs and one such sequence has been identified at position 990 of pBR322 (18). Linearised pBR322 was used as a substrate to evaluate the cleavage pattern of *M.smegmatis* DNA gyrase and compare it with the pattern obtained with the *E.coli* enzyme. The *M.smegmatis* enzyme cleavage pattern was similar to that of *E.coli* gyrase (Fig. 5A). To precisely map the ciprofloxacin-induced cleavage, a 240 bp DNA fragment containing pBR322 SGS was used (Fig. 5B and C). The position of DNA cleavage by mycobacterial DNA gyrase (Fig. 5D) was identical to that of *E.coli* (18). The enzyme cuts double-stranded DNA at a sequence AT↓GGCC, generating a four base overhang (Fig. 5D).

Effect of inhibitors

The effect of moxifloxacin on supercoiling activity of *E.coli* and *M.smegmatis* DNA gyrases was compared with that of ciprofloxacin (Table 1). Moxifloxacin was 2–3-fold more effective on the *M.smegmatis* DNA gyrase as compared with ciprofloxacin (Fig. 6A). In contrast, the difference in the inhibitory effects of the two drugs on *E.coli* DNA gyrase was marginal; the enzyme appears to be more sensitive to ciprofloxacin (Fig. 6B). This differential susceptibility of the mycobacterial enzyme to two fluoroquinolones indicates subtle differences in the ternary complexes of DNA–drug–gyrase. Further, these results also suggest the possibility that ciprofloxacin-resistant mycobacterial DNA gyrase could be sensitive to moxifloxacin. To test this hypothesis, the effect of moxifloxacin on DNA gyrase purified from ciprofloxacin-resistant *M.smegmatis* was studied. As expected, ciprofloxacin,

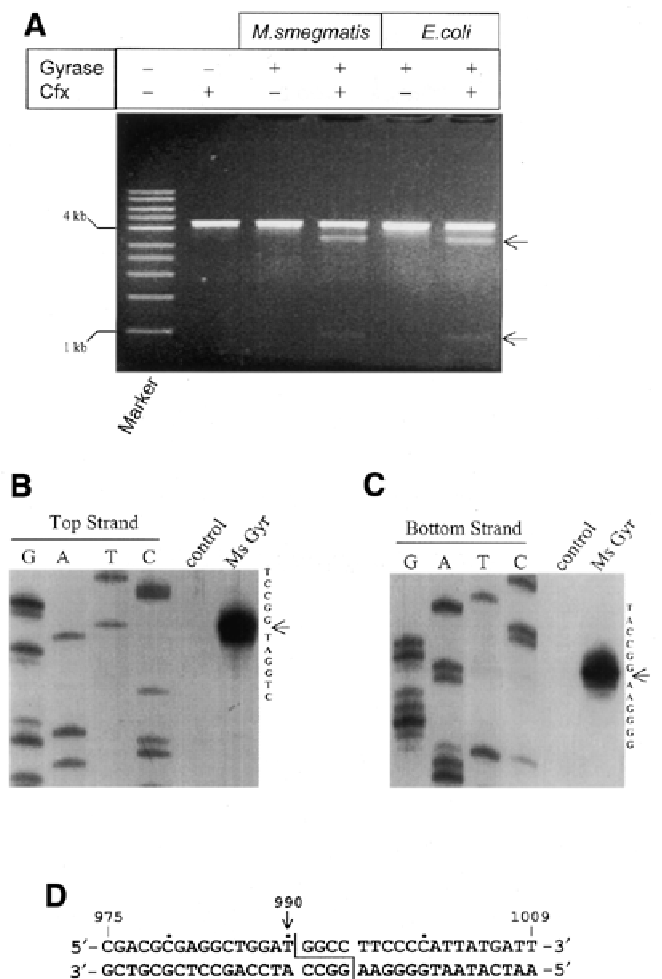

Figure 5. DNA cleavage site mapping of *M.smegmatis* DNA gyrase. (**A**) A comparison of ciprofloxacin-induced DNA cleavage pattern of linearised pBR322 between *E.coli* and *M.smegmatis* DNA gyrases. Marker, a 1 kb DNA ladder (Sigma). Arrows indicate the cleaved products (0.9 and 3.4 kb). (**B** and **C**) Precise mapping of cleavage position. Cleavage site in the top (B) and bottom (C) strands of the 240 bp DNA fragment of pBR322 encompassing the SGS. Cleavage reactions were performed with labelled DNA fragments in the presence of ciprofloxacin (30 µg ml^{-1}). G, A, T and C are sequencing lanes; lanes 5 and 6 are cleavage reactions in the absence and presence of *M.smegmatis* DNA gyrase, respectively. (**D**) Mycobacterial DNA gyrase cleavage positions in pBR322. Nucleotides are numbered showing the cleavage site position at 990.

even at a concentration of 800 µg ml^{-1}, did not inhibit enzyme activity. In contrast, the supercoiling activity was inhibited in the presence of 50 µg ml^{-1} moxifloxacin (Fig. 6C).

DISCUSSION

Being an essential enzyme, DNA gyrase is conserved in all bacteria and has retained the domainal organisation and motifs important for catalysis and function. However, there must be species-specific differences in the catalytic properties and efficiency of the enzyme from different sources. The enzyme activity has to be optimised for its physiological function and this aspect has not been addressed so far. In this manuscript we describe the characterisation of DNA gyrase from mycobacteria to reveal the differences in activity with respect to the prototype *E.coli* enzyme. A GyrB-specific monoclonal antibody was used as an affinity handle to purify *M.smegmatis* DNA gyrase. The procedure allows rapid purification of individual subunits with high specific activity. The reconstituted heterotetrameric enzyme exhibited reaction characteristics somewhat similar to that of *E.coli* gyrase, such as Mg^{2+}- and ATP-dependent DNA supercoiling and ATP-independent

Table 1. Inhibition of DNA supercoiling by ciprofloxacin and moxifloxacin

DNA gyrase	IC_{50} (µg ml^{-1})	
	Ciprofloxacin	Moxifloxacin
Escherichia coli	0.3	0.4
Mycobacterium smegmatis	10	4
Mycobacterium smegmatis (Cfx^R)	>800	50

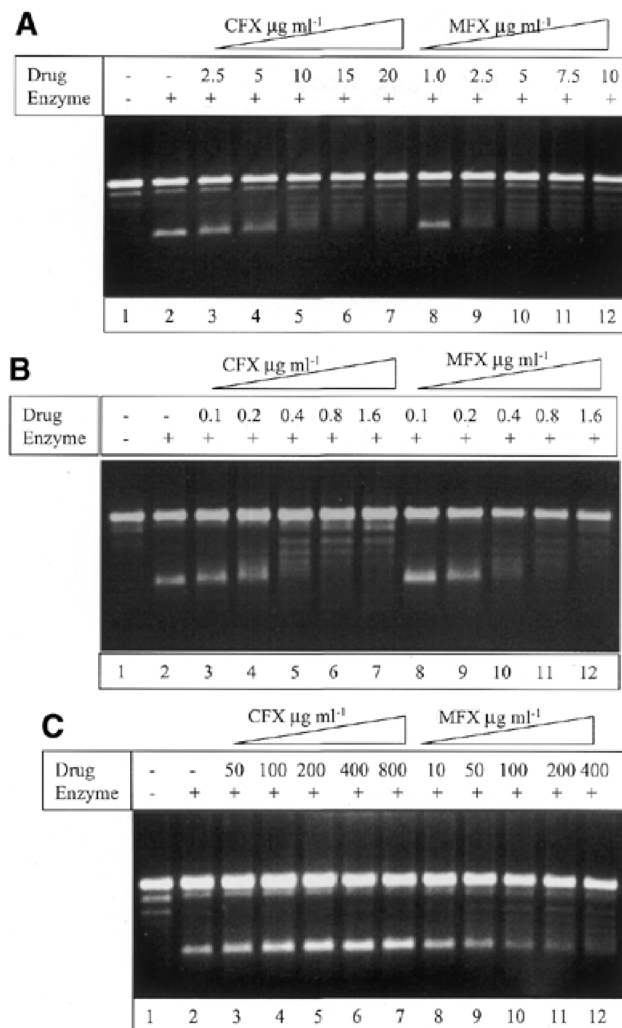

Figure 6. Inhibition of supercoiling activity of *M.smegmatis* and *E.coli* DNA gyrase. Effect of fluoroquinolones on the supercoiling activity of wild-type *M.smegmatis* (**A**), *E.coli* (**B**) and ciprofloxacin-resistant *M.smegmatis* (**C**) DNA gyrase. Supercoiling assays were carried out in the presence of varying concentrations of ciprofloxacin (lanes 3–7) and moxifloxacin (lanes 8–12). Drugs were added at the time of reaction initiation. Lane 1, relaxed pUC18 DNA; lane 2, supercoiling reaction in the absence of drug.

DNA relaxation activities. However, the enzyme exhibited significant differences with respect to decatenation, ATP hydrolysis, DNA-binding characteristics, catalytic efficiency and susceptibility to certain quinolone class drugs.

Mycobacterium smegmatis DNA gyrase is a better decatenase than *E.coli* gyrase at physiologically relevant Mg^{2+} concentrations. Multiply linked DNA dimers arise during DNA replication when the replication forks proceed through the residual non-replicated parental duplex present in late Cairns-type replication intermediates without concomitant unlinking of parental strands (34). Once the nascent strands are sealed, these catenated DNA dimers could then be resolved into monomer rings by the action of a type II topoisomerase (35). *Escherichia coli* has two type II topoisomerases, i.e. DNA gyrase (22) and topoisomerase IV (36), each having distinct roles *in vivo*. DNA gyrase is responsible for the generation of negative supercoils and is essential for chromosome condensation leading to proper chromosome partitioning during cell division (37,38). Topoisomerase IV is the key decatenating enzyme carrying out the actual separation of catenated daughter chromosomes (39,40). Although DNA gyrase and topoisomerase IV are closely related, they differ strikingly in their ability to catalyse inter versus intramolecular strand passage reactions. Unlike the mycobacterial gyrase, the *E.coli* enzyme is a poor decatenase (Fig. 2B and C) (24,25). The relatively weak decatenation activity of *E.coli* DNA gyrase was viewed as an inevitable consequence of its remarkable supercoiling activity (25). In contrast to the *E.coli* genome, many other bacterial genomes encode the barest minimum enzymatic components to maintain DNA topology. Only DNA gyrase and topoisomerase I are found in the genomes of *M.tuberculosis*, *Campylobacter jejuni*, *Deinococcus radiodurans* and *Treponema pallidum* (2). Some of these organisms may not require extremely efficient topoisomerase IV decatenase activity due to a slow growth nature and possible lower rates of replication. Organisms with a minimum topoisomerase make-up in their genomes would inevitably have evolved to retain reasonably efficient supercoiling and decatenation activities necessary for cell survival. Alternatively, the optimal intra and interstrand strand passage efficiency exhibited by mycobacterial gyrase could be a typical characteristic of many type II topoisomerases which do not exhibit a clear division of labour.

Intrinsic ATPase activity was not detectable in the case of *M.smegmatis* GyrB. The addition of GyrA alone led to some stimulation, while significant stimulation was observed only in the presence of both GyrA and DNA. DNA-stimulated ATP hydrolysis is a typical characteristic of DNA gyrase (31,41). There are some differences in the ATPase activity measured for the *E.coli* enzyme, which could be due to the purification protocols and the assay conditions employed. Staudenbauer and Orr (20) observed an appreciable level of intrinsic ATPase activity for GyrB that was unaltered by the addition of GyrA. In contrast, significant intrinsic ATPase activity was not observed in other studies (27,31). Addition of GyrA resulted in a low-level stimulation of ATPase activity (27,41). Further, the DNA-stimulated ATPase activity of the *E.coli* holoenzyme varied from 2- to 100-fold (20,31) with a range of values for K_m (0.2–0.45 mM) and k_{cat} (0.67–1.37 s^{-1}) (31). In our analysis, *M.smegmatis* DNA gyrase exhibited a lower affinity for ATP (0.68 mM) and slow enzyme turnover (k_{cat} = 0.39 s^{-1}) compared with any of the values measured for the *E.coli* enzyme.

The sequence analysis of DNA gyrase from different bacteria revealed that the C-terminal domain of GyrA, responsible for the DNA wrapping function, is most varied. The lower affinity of the *M.smegmatis* enzyme for DNA is not a surprising property considering the differences in the amino acid sequences of the various bacterial DNA gyrases. The differences in affinity for DNA could also be attributed to another aspect. The additional 165 amino acid stretch present in GyrB of *E.coli* is an absolute requirement for the holoenzyme to bind DNA (5). In contrast, mycobacterial DNA gyrase is a 'natural deletant' for this stretch, employing other regions of GyrB for DNA binding. The lower affinity for ATP in DNA-stimulated ATP hydrolysis exhibited by the enzyme might be due to the lower affinity of the gyrase for DNA. There is evidence which suggests that T-segment binding is required for DNA-stimulated ATPase activity (4). Capture of DNA inside the ATP-operated clamp may facilitate allosteric changes in the ATPase domain, which in turn may affect ATPase activity.

The lower affinity for DNA and slower rate of ATP hydrolysis would imply reduced supercoiling activity of *M.smegmatis* DNA gyrase. Slower supercoiling activity has also been observed with DNA gyrase from *Mycobacterium bovis* (42). The intrinsic low efficiency of mycobacterial gyrases raises intriguing questions about their *in vivo* function. The enzyme activity measured could be the actual rates at which it functions ahead of the replication fork and transcription bubble. Alternatively, the activity of the enzyme might be enhanced *in vivo* by other modulating factors. *Mycobacterium smegmatis* is a relatively slow growing bacterium and the rate of replication is also ~3-fold slower as compared with that of *E.coli* (43). There has to be an orchestrated movement of the replication machinery along with DNA gyrase operating ahead of the replication fork. Thus the two times slower replication rate and the corresponding reduction in gyrase activity seem to be a case for functional optimisation.

Fluoroquinolones, derivatives of classic quinolones, exhibit enhanced antibacterial and pharmacokinetic properties and are proven drugs against a variety of bacterial infections. Although mycobacteria have been found to be naturally less susceptible to quinolones than many other bacteria (44), new quinolones have been demonstrated to be active against mycobacterial infections (45). Amongst them, moxifloxacin has been found to be the most active compound against mycobacteria (46) and the present studies reveal that moxifloxacin is 2–3-fold more effective than ciprofloxacin in inhibiting *M.smegmatis* DNA gyrase supercoiling activity. In contrast, such a marked difference was not observed with the *E.coli* enzyme. This variation in quinolone susceptibility between the two enzymes indicates the differences in the respective enzyme–drug–DNA ternary complexes. At the molecular level, the lower susceptibility of DNA gyrase to quinolones is assigned to alterations in the quinolone resistance-determining region (QRDR) of GyrA, which is supposed to be the site of interaction between the A subunit and quinolones (47). Ser83 and Asp87 of the *E.coli* GyrA QRDR region are involved in gyrase–drug–DNA interactions. However, in many mycobacterial species Ser83 of the *E.coli* QRDR is replaced by Ala at the corresponding position, and the lower susceptibility of mycobacteria to quinolones is

attributed to this change (44). The results presented in Figure 6C substantiate the differential susceptibility of the mycobacterial enzyme to two different fluoroquinolones. Moxifloxacin has been found to be effective against wild-type as well as ciprofloxacin-resistant mycobacterial DNA gyrase. Thus moxifloxacin, a C-8 methoxy quinolone, must be forming altered and/or additional contacts with *M.smegmatis* DNA gyrase in the ternary complex. These results reveal the potential to develop new fluoroquinolones which do not extend cross-resistance to the existing ones. Since moxifloxacin has also been found to be a poor substrate for active efflux (48), it can serve as a potential lead molecule to design new ligands with highly specific antimycobacterial activity. Such rational molecular design is timely to counter the emerging multidrug-resistant mycobacterial infections.

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