Eur. J. Biochem. **268**, 2038–2046 (2001) © FEBS 2001

Monoclonal antibodies to mycobacterial DNA gyrase A inhibit DNA supercoiling activity

U. H. Manjunatha[1], S. Mahadevan[1], Sandhya S. Visweswariah[2] and V. Nagaraja[1]

[1]*Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India;* [2]*Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore, India*

DNA gyrase is an essential type II topoisomerase found in bacteria. We have previously characterized DNA gyrase from *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. In this study, several monoclonal antibodies were generated against the gyrase A subunit (GyrA) of *M. smegmatis*. Three, MsGyrA:C3, MsGyrA:H11 and MsGyrA:E9, were further analyzed for their interaction with the enzyme. The monoclonal antibodies showed high degree of cross-reactivity with both fast-growing and slow-growing mycobacteria. In contrast, none recognized *Escherichia coli* GyrA. All the three monoclonal antibodies were of IgG_1 isotype falling into two distinct types with respect to epitope recognition and interaction with the enzyme. MsGyrA:C3 and MsGyrA:H11 IgG, and their respective Fab fragments, inhibited the DNA supercoiling activity catalyzed by mycobacterial DNA gyrase. The epitope for the neutralizing monoclonal antibodies appeared to involve the region towards the N-terminus (residues 351–415) of the enzyme in a conformation-dependent manner. These monoclonal antibodies would serve as valuable tools for structure–function analysis and immunocytological studies of mycobacterial DNA gyrase. In addition, they would be useful for designing peptide inhibitors against DNA gyrase.

Keywords: DNA gyrase; enzyme inhibition; epitope mapping; monoclonal antibody; mycobacterium.

The topological state of DNA plays an important role in biological activity and is maintained by a group of enzymes called topoisomerases [1,2]. These enzymes change the topology of DNA by passing one segment through another. The type I enzymes catalyze DNA-strand passage by transiently breaking one strand at a time and type II enzymes create transient breaks in both strands of a DNA segment for the enzyme-mediated passage of the second segment [1–3]. Type II topoisomerases are essential cellular enzymes that function in the segregation of newly replicated chromosome pairs, chromosome condensation and in altering DNA superhelicity [4]. DNA gyrase is a type II topoisomerase exclusively found in prokaryotes. It is the only enzyme that can introduce negative supercoiling, in a reaction that depends on ATP hydrolysis. The enzyme also catalyzes a number of other topological interconversions such as knotting–unknotting and catenation–decatenation [3].

DNA gyrase from *Escherichia coli* is composed of two subunits, A (GyrA) and B (GyrB). The active enzyme is a tetramer of two subunits each of GyrA and GyrB [5,6]. The GyrA subunit has the active site for DNA binding, cleavage and resealing, whereas GyrB powers the reaction by catalyzing ATP hydrolysis [7]. DNA gyrase is the target of two distinct classes of inhibitors, coumarins and quinolones. Coumarins bind to GyrB and are competitive inhibitors with respect to ATP. In contrast, quinolones bind DNA gyrase when the enzyme is complexed with DNA and trap the enzyme in an abortive ternary complex [8].

Of the 50 species in the genus mycobacterium, about 20 are of medical importance. Apart from well-known human mycobacterial pathogens, such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*, which cause tuberculosis and leprosy, other mycobacteria such as *Mycobacterium avium*, *Mycobacterium kansasii*, *Mycobacterium chelonae* and *Mycobacterium fortuitum* can cause opportunistic infections in immunocompromised hosts [9]. The emergence of multidrug-resistant strains of *M. tuberculosis* has resulted in the urgent need for new drugs to combat *M. tuberculosis* infections [10]. To address the role of DNA gyrase in mycobacteria and with a view to develop it as a suitable molecular target for antimycobacterials, we have initiated studies on the molecular characterization of DNA gyrase from *Mycobacterium smegmatis* [11] and *M. tuberculosis* [12].

Monoclonal antibodies (mAbs) have become powerful tools in biology due to the specificity of interaction with their target molecules leading to a variety of applications. They are routinely used in diagnostics, structure–function analyses and as reagents to study protein–protein interactions. For example, Nemoto and coworkers have used a panel of anti-HSP90 mAbs to describe domain structures and immunogenic regions of heat shock protein, HSP90 [13]. The regions of interaction of UvrB helicase with various other components of the nucleotide excision repair pathway were dissected using UvrB-specific mAbs [14]. A more recent application is the identification of peptide

Correspondence to V. Nagaraja, Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, 560 012, India. Fax: + 91 80 360 2697, Tel.: + 91 80 360 0668, E-mail: vraj@mcbl.iisc.ernet.in

Abbreviations: GyrA, DNA gyrase A subunit; IPTG, isopropyl thio-β-D-galactoside; VEGF, vascular endothelial growth factor; GST, glutathione S-transferase.

Enzyme: DNA gyrase (EC 5.99.1.3).

(Received 29 August 2000, revised 21 November 2000, accepted 6 February 2001)

inhibitors for potential antigens using a mAb approach. Tournaire *et al*. [15] have identified a peptide that blocks vascular endothelial growth factor (VEGF)-mediated angiogenesis, using an anti-VEGF mAb. In this study, we report the generation of a panel of *M. smegmatis* GyrA-specific mAbs and their interaction with mycobacterial DNA gyrase.

MATERIALS AND METHODS

Bacterial strains and materials

M. smegmatis SN2 and *E. coli* DH10B were laboratory strains. *M. tuberculosis* H37Rv was obtained from the National Tuberculosis Institute, Bangalore, India. *M. avium*, *Mycobacterium bovis*, *M. kansasii, M. fortuitim, Mycobacterium gilvum*, *Mycobacterium phlei* and *Mycobacterium voccae* were provided by V.M. Katoch, Central JALMA Institute for Leprosy, Agra, India. *M. leprae* cell lysate was a kind gift from P.J. Brennan, Colorado State University, CO, USA. All mycobacterial species were grown in modified Youmans and Karlson's medium with 0.2% Tween 80 at 37 °C [16] and cell-free extracts were prepared as described earlier [17]. Protein was estimated by the method of Bradford [18]. Enzyme-conjugated anti-mouse and anti-rabbit Ig and the enhanced chemiluminescence Kit were from Amersham Pharmacia Biotech, UK. Iscove's Modified Dulbecco's Medium, adjuvants and fetal bovine serum were from Gibco Life Technologies (Gaithersburg, MD, USA).

Construction of plasmids with truncated GyrA

A list of plasmids used in this study is presented in Table 1. To generate pGyrA:81–938 (a clone representing residues 81–938) of *M. tuberculosis* GyrA, plasmid pMN6R [12] (Table 1) was digested with *Ase*I and end filled with the Klenow fragment of DNA polymerase I, followed by *Nco*I digestion. The released 850-bp fragment was ligated into *Nco*I- and *Sma*I-digested pTrc99C. The rest of the *gyrA* deletion clones shown in Table 1 were constructed using plasmid pMK17 which encodes full-length GyrA from *M. tuberculosis* [17]. Plasmid pMK17 was digested with *Sal*I and the 5.3-kb fragment was re-ligated to obtain the pGyrA:1–351/767–938 deletion construct (a GyrA clone with 352–766 amino-acid residues deleted). For pGyrA:1–90/415–938 generation (N-terminal deletion of GyrA from 91 to 414 amino-acid residues), plasmid pMK17 was digested with *Mlu*I and end filled with the Klenow fragment of DNA polymerase I, followed by *Eco*RV digestion. The 5.5-kb fragment containing a part of GyrA was re-ligated and used for overexpression. To generate the clone encoding 70 amino acids between 344 and 415, oligonucleotide primers for PCR amplification were designed with *Sal*I restriction site (forward primer: CTAGCCATGGTCGACGGGGTG and reverse primer: TCTTTAGTCGACGGTCTCCGACGC). The PCR product obtained with the pMK17 template was digested with *Sal*I restriction endonuclease and cloned into pGEX-5X-3 digested with *Sal*I to obtain pGST-GyrA:351–415 plasmid. The cloned DNA fragment was sequenced by the chain termination method using reverse primer.

Expression and purification of proteins

GyrA of *M. tuberculosis* was overexpressed in BL21(DE3)-pLysS cells harboring pMK17 [17]. Overexpressed protein was purified by electroelution from preparative SDS/PAGE and antiserum was raised in rabbits as described earlier [17]. For the purification of *M. smegmatis* GyrA, *E. coli* strain BL21(DE3)pLysS harboring the plasmid pMK26 (MsGyrA) [11] was grown in Luria-Bertani broth at 37 °C. At an A_{600} of 0.6, the cells were induced with 500 μM isopropyl thio-β-D-galactoside (IPTG) for 3 h at 18 °C. Cells were collected by centrifugation, resuspended in sonication buffer (20 mM Tris pH 8.0, 1 mM EDTA, 10% glycerol, 5 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine HCl and 1% Triton X-100) and lyzed by sonication. The cell lysate was centrifuged at 100 000 ***g*** to obtain the cell-free extract, which was treated with ammonium sulfate to obtain a 30–55% pellet fraction. The pellet was solubilized in 50 mM Tris-HCl pH 7.6, 10% glycerol, 2 mM EDTA and 2 mM 2-mercaptoethanol (TGEM buffer) and subjected to purification on MonoQ and heparin-Sepharose columns using an NaCl gradient elution; the GyrA protein eluted at 300–400 mM NaCl. *E. coli* GyrA was purified from DH10B cells harboring pPH3 [19] plasmid containing the *E. coli gyrA* gene, as described by Maxwell and Howells [20].

Purification of DNA gyrase from *M. smegmatis* SN2

DNA gyrase from *M. smegmatis* was purified by novobiocin-Sepharose column, as described [21] with the following

Table 1 Plasmids used in this study. GST, glutathione S-transferase.

Plasmid	Description	Reference
pMK26	pET11d carrying *M. smegmatis gyrA*	[11]
pMN6R	*M. tuberculosis gyrA* in pUC19	[12]
pMK17	pET20b$^+$ carrying *M. tuberculosis gyrA*	[17]
pPH3	*E. coli gyrA* overexpression plasmid	[19]
pGyrA:81–938	*gyrA* deletion construct	This study
pGyrA:1–351/767–938	*gyrA* deletion construct	This study
pGyrA:1–90/415–938	*gyrA* deletion construct	This study
pGST-GyrA:351–415	*gyrA* deletion construct	This study
pTrc99C	Expression vector with trp-lac hybrid promoter	Amersham Pharmacia Biotech
pGEX-5X-3	GST fusion expression vector	Amersham Pharmacia Biotech

modifications. During purification, TGEM buffer was used and the purified protein fractions were stored in TGEM buffer with 20% glycerol.

Generation of mAbs against GyrA

Balb/c mice were immunized intradermally with 100 μg of *M. smegmatis* GyrA and boosted three times with 50 μg of *M. smegmatis* GyrA in Freund's incomplete adjuvant at intervals of one month. Four days prior to fusion, the mice were administered 25 μg of antigen intraperitoneally. Cell fusion was performed with the myeloma cell line Sp2/O Ag-14 [22] as previously described [23]. Wells containing hybrid clones were screened for the presence of GyrA-specific antibodies 15 days after fusion by ELISA [24], using *M. smegmatis* GyrA (2 $\mu g \cdot mL^{-1}$) coated on ELISA plates (Nunc) in 10 mM sodium phosphate, pH 7.2, containing 0.9% NaCl. Plates were blocked with BSA (0.4%) in NaCl/P_i. Antibody-secreting clones were subcloned and monoclonality was established for eight clones by limiting dilution. Three antibody-secreting clones MsGyrA:C3, MsGyrA:H11 and MsGyrA:E9 were further characterized. Ascitic fluid was prepared by intraperitoneal injection of 1×10^6 hybridoma cells into Balb/c mice. Immunoglobulins were purified from ascitic fluid using protein A-Sepharose affinity column chromatography [25]. To study the effect of mAbs on supercoiling activity, mAbs were dialyzed against 35 mM Tris/HCl, pH 7.4, 50 mM KCl in 10% glycerol. The Ig isotyping was carried out using a kit from Sigma according to the manufacturer's instructions and three mAbs were isotyped as IgG_1.

Fab fragments of the IgG were prepared by digestion of the purified IgG by papain as described earlier [26], purified by protein A-Sepharose affinity column and analyzed by Superdex-200 column chromatography. For Western blot analysis, proteins were resolved on an 8% SDS/PAGE [27]. Proteins were transferred to polyvinylidine difluoride membrane in transfer buffer (50 mM Tris/glycine buffer, pH 8.5, containing 20% methanol) for 3 h at 200 mA. The membrane was blocked with NaCl/P_i containing 2% BSA and probed with either polyclonal or monoclonal antibodies. Bound IgG was detected using sheep anti-(mouse IgG) Ig or donkey anti-(rabbit IgG) Ig conjugated to horseradish peroxidase and enhanced chemiluminescence reagents (Amersham-Pharmacia Biotech).

Immunoprecipitations

DNA gyrase (5 μg) was preincubated with 100 nM of mAbs (MsGyrA:C3 or MsGyrA:H11) or 1 μM of MsGyrA:E9 or normal mouse Ig in 35 mM Tris/HCl pH 7.6, 2 mM $MgCl_2$, 100 mM potassium glutamate, 0.14 mM EDTA, 1.8 mM spermidine HCl, 6.5% glycerol, 5 mM dithiothreitol, 9 $\mu g \cdot mL^{-1}$ yeast tRNA, 50 $\mu g \cdot mL^{-1}$ BSA, 1.4 mM ATP (KGB buffer) at 4 °C for 2 h. The immunocomplex was precipitated by adding 10 μL of protein G-agarose and centrifuging at 20 000 ***g*** for 2 min at 4 °C. The supernatants from immunoprecipitation reactions were assayed for supercoiling activity, and after extensive washing, the pellets were subjected to Western blotting, as described earlier. For immunoprecipitations with MsGyrA:E9 under denaturing conditions, DNA gyrase was treated with 1% SDS in immunoprecipitation buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 5 mM 2-mercaptoethanol, 50 mM NaCl, 1% Nonidet P-40 and 5% glycerol) for 2 h at 4 °C. 100 $\mu g \cdot mL^{-1}$ MsGyrA:E9 was added, the immunoprecipitates were washed and Western blotting was performed with GyrA polyclonal antibodies as described earlier.

DNA gyrase supercoiling assay

Relaxed DNA was prepared by treating supercoiled pUC18 DNA with purified *E. coli* topoisomerase I [28]. The supercoiling reaction with *M. smegmatis* DNA gyrase was performed as described earlier [11], with some modifications. The reaction was carried out in potassium glutamate buffer (KGB) in a 20-μL reaction volume with 200 ng of relaxed DNA. Potassium glutamate was necessary for the optimal activity of the enzyme. After 30 min of incubation at 37 °C, the reaction was stopped by adding 4 μL of stop buffer (0.6% SDS, 0.2% bromophenol blue, 0.2% xylene cyanol FF) and heat inactivation at 75 °C for 15 min. The samples were electrophoresed in a 1.2% agarose gel for 10–12 h at 25 V. The gel was stained with ethidium bromide (0.5 $\mu g \cdot mL^{-1}$). The supercoiling assays with *E. coli* gyrase were carried out as described by Mizuuchi *et al.* [29].

RESULTS

Generation and characterization of monoclonal antibodies to DNA GyrA

A panel of eight mAbs against *M. smegmatis* GyrA was obtained. When tested on ELISA with purified *M. tuberculosis* and *E. coli* GyrA, all cross-reacted with *M. tuberculosis* GyrA to a similar extent but none recognized *E. coli* GyrA. Western blot analysis using the three mAbs MsGyrA:C3, MsGyrA:H11 and MsGyrA:E9 was performed with purified proteins and cell lysates prepared from *M. smegmatis* SN2, *M. tuberculosis* H37Rv and *E. coli* DH10B. The antibodies recognized purified recombinant mycobacterial GyrA subunits and GyrA from whole cell-free extracts, but not from *E. coli* (Fig. 1B and C). Recombinant and wild-type GyrA from both *M. tuberculosis* and *M. smegmatis* show proteolytic degradation to a protein fragment of 78 kDa, which was also recognized by the mAbs. Preliminary studies employing surface plasmon resonance spectroscopy indicated that mAbs interact with GyrA with high affinity (data not shown).

Sequence alignment of *M. smegmatis* GyrA (X84077 [11]), with other known mycobacterial DNA GyrA subunits (*M. tuberculosis*, L27512 [30] and *M. leprae*, Q57532 [31]) indicates approximately 90% sequence identity at the amino-acid level [17]. To study the cross-reactivity of the mAbs with gyrases from other mycobacterial species, crude cell lysates were prepared and analyzed by Western blotting. All the three mAbs recognized GyrA from both fast-growing and slow-growing mycobacterial species

The GyrA subunits of all the species tested have very similar sizes (Fig. 2). Although *M. leprae* GyrA has been shown to contain an intein [31], in our Western blot analysis, only the mature GyrA with a comparable size to that of other mycobacterial species was detected.

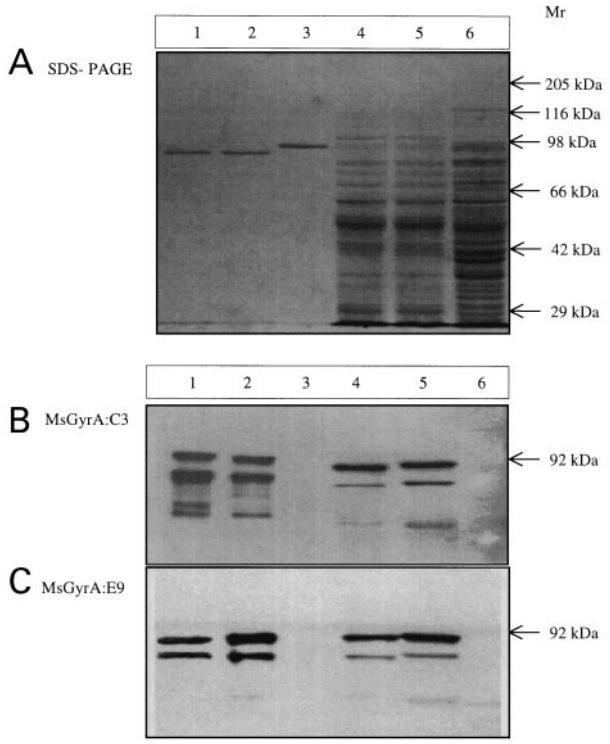

Fig. 1. Characterization of the anti-GyrA mAbs. Purified GyrA proteins (1 μg·lane^{-1}) or cell-free extracts (20 μg·lane^{-1}) of *M. tuberculosis* (lanes 1 and 4), *M. smegmatis* (lanes 2 and 5) and *E. coli* (lanes 3 and 6) were run on SDS/PAGE and then probed with the mAbs on Western blot. (A) SDS/PAGE. (B) Western blot analysis using MsGyrA:C3 as a probe (10 ng·mL^{-1}). (C) Western blot analysis using MsGyrA:E9 as a probe (1 μg·mL^{-1}).

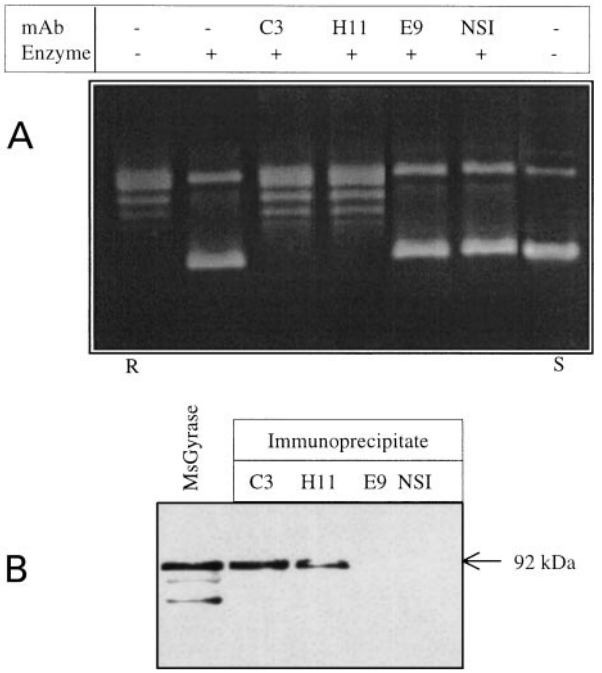

Fig. 3. Immunoprecipitation of mycobacterial DNA gyrase holoenzyme. *M. smegmatis* DNA gyrase was incubated with 200 nM of anti-GyrA mAb in KGB buffer on ice for 30 min. Immunocomplexes were recovered using 10 μL protein G-agarose. (A) The supernatant of the immunoprecipitation reaction was assayed for DNA supercoiling activity. Nonspecific IgG was used as negative control. (B) Proteins bound to the protein G beads were analyzed by SDS/PAGE and Western blotting was performed with *M. tuberculosis* GyrA polyclonal antibody at a 1 : 20 000 dilution. NSI, nonspecific IgG; R, relaxed pUC18 DNA; S, supercoiled pUC18 DNA.

Interaction of mAbs with DNA gyrase in its native conformation

As the mAbs were generated against the GyrA subunit alone, and the functional holoenzyme comprises A and B subunits [5,6,32], we studied the interaction of the mAbs with preparations of the holoenzyme from *M. smegmatis*. DNA gyrase from *M. smegmatis* was treated with the various mAbs, and antibody-bound enzyme was immunoprecipitated

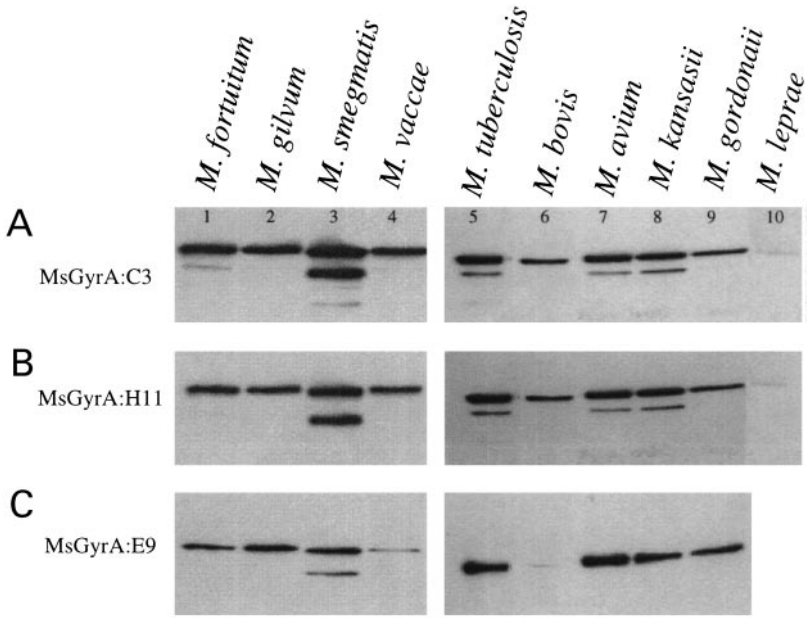

Fig. 2. Cross-reactivity of anti-GyrA mAbs with various mycobacterial species. 20 μg of fast-growing (lanes 1–4) and slow-growing (lanes 5–10) mycobacterial cell-free extracts were subjected to SDS/PAGE. After transferring proteins to the polyvinylidine difluoride membrane, the blots were probed with the mAbs. Proteolytic degradation of GyrA to a 78 kDa species was observed in some cases. (A) MsGyrA:C3 (10 ng·mL^{-1}). (B) MsGyrA:H11 (10 ng·mL^{-1}). (C) MsGyrA:E9 (1 μg·mL^{-1}).

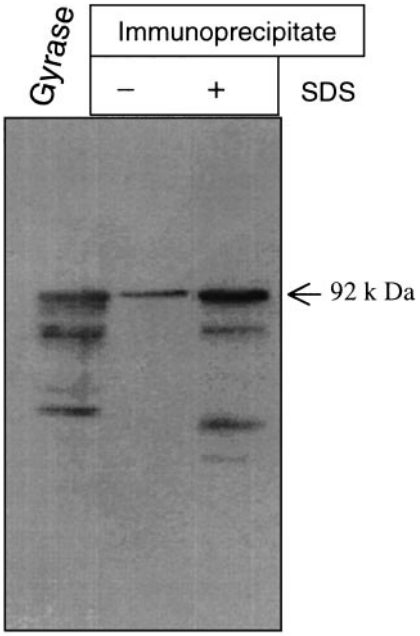

Fig. 4. Western blot analysis of MsGyrA:E9 immunoprecipitate with anti-GyrA polyclonal antibodies (1 : 20 000 dilution). DNA gyrase was immunoprecipitated with 100 μg·mL^{-1} MsGyrA:E9 with or without prior treatment with 1% SDS for 2 h. Immunocomplexes were recovered using protein G-agarose and bound proteins were analyzed by SDS/PAGE and Western blotting.

with protein G-agarose. Both MsGyrA:C3 and MsGyrA:H11 could immunoprecipitate the gyrase activity but gyrase activity was detected in the supernatant of the immunoprecipitate formed with MsGyrA:E9 (Fig. 3A), indicating that MsGyrA:E9 could not interact with the holoenzyme. Analysis of the presence of enzyme in the immunoprecipitates and supernatants by Western blot analysis confirmed these observations (Fig. 3B). These results suggested that the epitope for MsGyrA:E9 was masked in the holoenzyme, either by the natural folding of the GyrA subunit or by its interaction with the GyrB subunit. Indeed, denaturation of the enzyme using SDS allowed immunoprecipitation of the enzyme by MsGyrA:E9 (Fig. 4).

Epitope mapping of MsGyrA mAbs

The initial epitope analysis was performed using surface plasmon resonance spectroscopy, where binding analysis of mAbs was performed on *M. smegmatis* GyrA immobilized on the biosensor CM5 surface (data not shown). The results indicated that binding of MsGyrA:C3 and MsGyrA:H11 on GyrA was mutually exclusive, suggesting either an identical recognition site for the two mAbs or interaction with two regions in the enzyme that were very close to each other. However, MsGyrA:E9 showed a different pattern of binding indicating that its epitope is distinct from the other two mAbs (data not shown).

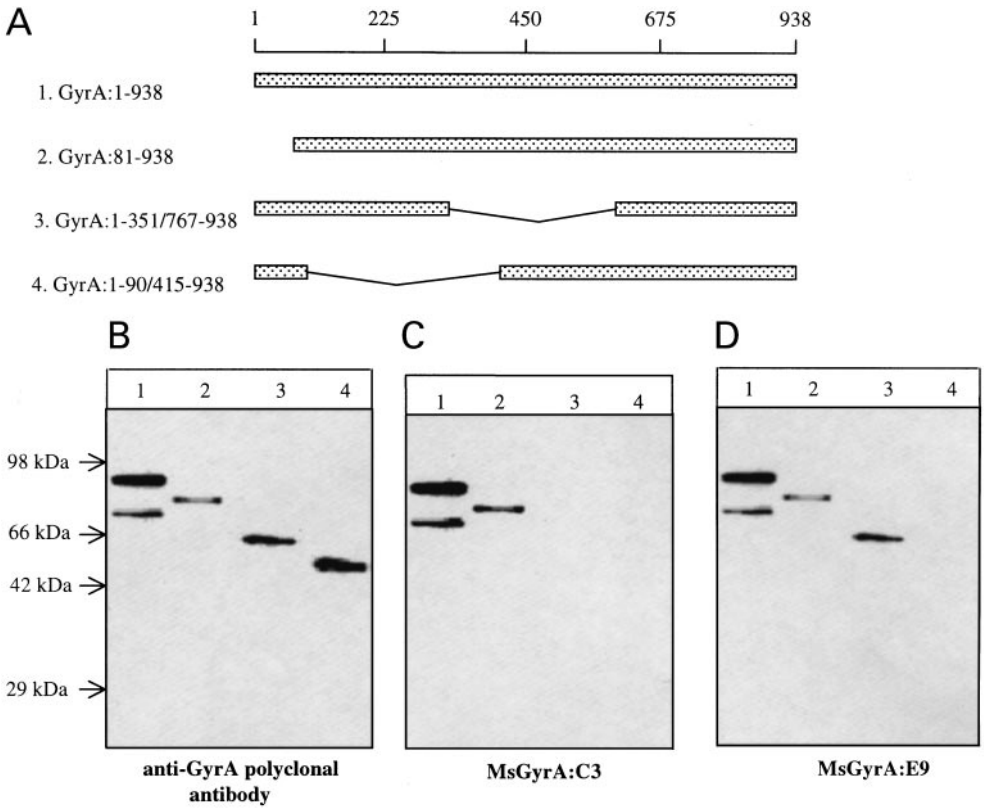

Fig. 5. Epitope mapping of anti-GyrA mAbs using deletion mutants. (A) Schematic representation of various GyrA truncated proteins. The number on the left side of the panel denotes the GyrA residues present in different proteins. (B–D) Immunoblot analysis of truncated *M. tuberculosis* GyrA proteins. Extracts from *E. coli* cells (20 μg) expressing truncated GyrA proteins were separated by 10% SDS/PAGE. After transferring proteins to the polyvinylidine difluoride membrane, the blots were probed with antibody. (B) Polyclonal anti-GyrA antibody (1 : 20 000 dilution). (C) MsGyrA:C3 (10 ng·mL^{-1}). (D) MsGyrA:E9 (1 μg·mL^{-1}).

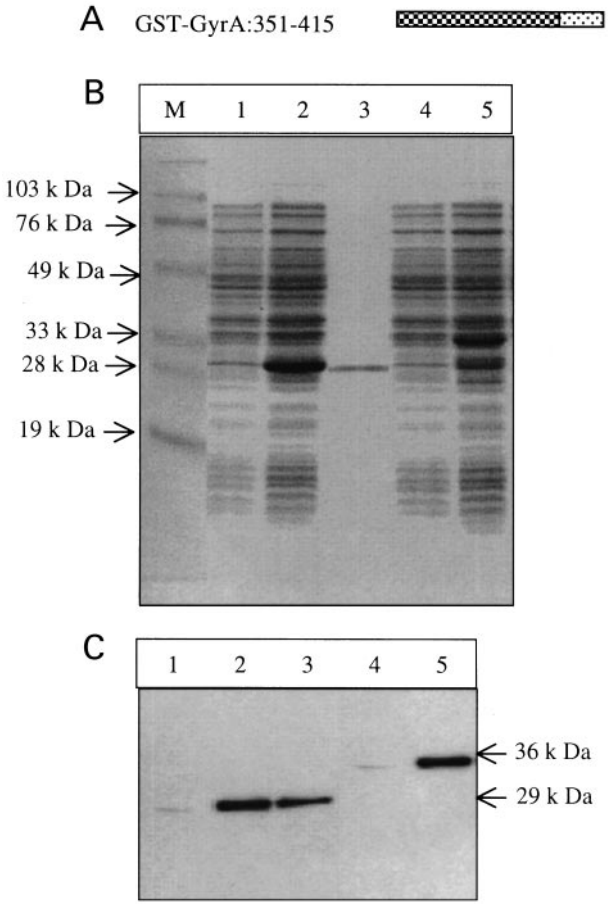

Fig. 6. Overexpression and interaction of GyrA:351–415 with MsGyrA:C3. (A) Diagrammatic representation of GyrA:351–415 as an N-terminal glutathione S-transferase fusion. (B) 12% SDS/PAGE and (C) Western blot analysis with anti-GST antibody. *E. coli* DH10b cell lysates harboring pGEX-5X-3 plasmid without induction (lane 1) or induced with 300 μM IPTG (lane 2). Lane 3 is 0.5 μg of purified glutathione S-transferase. *E. coli* cells harboring pGST-GyrAΔ uninduced (lane 4) and induced with 300 μM IPTG (lane 5). M, protein molecular mass markers.

To determine precise regions of GyrA–mAb interaction, Western blot analysis was performed with bacterial lysates prepared from cells expressing various fragments of the GyrA subunit. As shown in Fig. 5, deletion of the first 80 amino acids allowed interaction of all the three mAbs. However, MsGyrA:E9 did not interact with GyrA:1–90/415–938, but recognized GyrA:1–351/767–938. Therefore we conclude that the epitope recognized by MsGyrA:E9 lies within a 261 amino-acid segment located between amino acids 90 and 351. MsGyrA:C3 and H11 did not recognize GyrA:1–90/415–938 or GyrA:1–351/767–938, indicating that the epitope for these two mAbs could be located between amino acids 351 and 415. However, when this 65 amino-acid stretch was expressed in *E. coli* in a fusion with glutathione S-transferase (Fig. 6) neither MsGyrA:C3 nor H11 recognized the fusion protein, implying that MsGyrA:C3 and MsGyrA:H11 recognize a conformation-specific epitope.

Effect of interaction of mAb with DNA gyrase on enzyme activity

To test whether interaction of the enzyme with the mAbs affected DNA gyrase supercoiling activity, *M. smegmatis* gyrase was preincubated with individual mAbs to allow the formation of the antigen–antibody complex. The mixture was then added to relaxed pUC18 DNA, in supercoiling reaction as detailed in Materials and methods. The results (Fig. 7) showed that both MsGyrA:C3 and MsGyrA:H11 inhibited the mycobacterial DNA gyrase supercoiling activity while MsGyrA:E9 showed no reduction in supercoiling activity. None of the mAbs affected the supercoiling activity of *E. coli* DNA gyrase. This is in agreement with ELISA and Western blotting results, which indicated the absence of cross-reactivity with the *E. coli* enzyme.

As whole IgG was used in these studies, it was possible that inhibition of gyrase activity resulted from steric effects caused by the Fc regions of these mAbs or by cross-linking of adjacent gyrase molecules. To address this possibility, Fab fragments of mAbs were prepared and tested to determine whether these monovalent fragments were also capable of inhibiting DNA gyrase supercoiling activity. As shown in Fig. 8, Fab fragments of MsGyrA:C3 and MsGyrA:H11 also inhibited the supercoiling reaction. MsGyrA:C3 completely inhibited supercoiling activity at a concentration of 10 nM

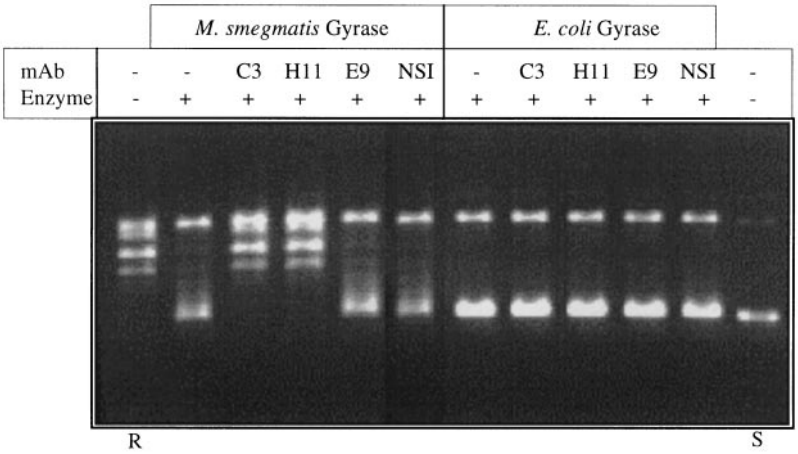

Fig. 7. Inhibition of DNA supercoiling activity of mycobacterial DNA gyrase by mAbs. DNA gyrase was preincubated with 1 μM of mAb on ice for 30 min. The supercoiling reaction buffer containing 300 ng of relaxed DNA was added and the mixture was incubated for 30 min at 37 °C. The reactions were terminated by adding stop buffer and heat inactivation for 15 min at 75 °C. The reaction products were separated on 1.2% agarose gels and the gel was stained with ethidium bromide (0.5 $\mu g \cdot mL^{-1}$). R, relaxed pUC18 DNA; S, supercoiled pUC18 DNA.

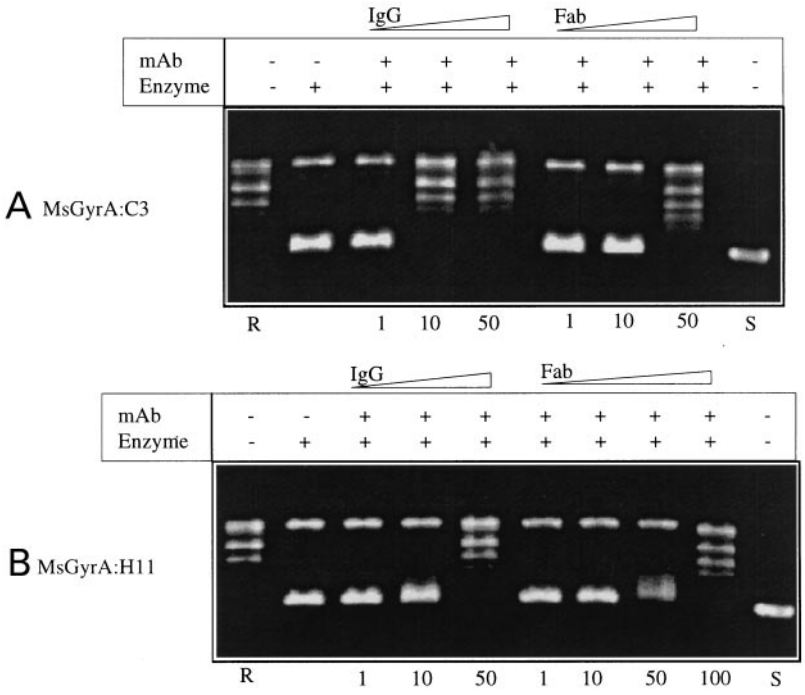

Fig. 8. Inhibition of mycobacterial DNA gyrase supercoiling activity by different concentrations of IgG and Fab fragments of mAbs. *M. smegmatis* DNA gyrase was preincubated with increasing amounts of Fab fragments of MsGyrA:C3 or MsGyrA:H11 on ice for 30 min. The assay conditions are as described in Materials and methods and the legend to Fig. 7. The concentrations of the mAbs are also indicated (nM).

IgG and 50 nM Fab, whereas MsGyrA:H11, the affinity of which is slightly lower than MsGyrA:C3, inhibited supercoiling activity completely at a concentration of 50 nM IgG and 100 nM Fab (Fig. 8).

DISCUSSION

In this study, we describe the generation of mAbs to *M. smegmatis* GyrA that exhibit remarkable specificity for GyrA from different mycobacteria. The epitopes for these mAbs have been mapped to different regions of the protein. Moreover two mAbs were shown to inhibit DNA supercoiling activity of the enzyme. The studies reported here are the first to describe the development and characterization of mAbs to mycobacterial DNA gyrase A subunit.

Sequence homology between DNA gyrase genes from diverse bacteria suggest that DNA gyrases from many bacteria share several structural and functional motifs that are involved in, for example, ATP binding and hydrolysis or DNA cleavage. [33]. However, the mAbs we have described here did not interact with *E. coli* gyrase, in spite of the fact that gyrase from *E. coli* and mycobacteria share several regions of extended sequence homology [34]. All recognized *M. tuberculosis* GyrA. Our earlier results had indicated that despite the 46.6% amino-acid identity of *E. coli* GyrA with mycobacterial GyrA, polyclonal antibodies raised to *M. tuberculosis* GyrA did not recognize the *E. coli* enzyme [17]. These results indicate that the major antigenic regions of mycobacterial gyrase reside outside the regions homologous in the *E. coli* enzyme.

Our results show that the mAbs have a relatively conservative pattern of cross-reactivity, and interaction was limited to GyrA from mycobacteria. The mAbs cross-reacted well with both fast-growing and slow-growing mycobacteria, indicating that GyrA may be similar among different mycobacterial species with respect to size and immunogenicity of the protein (Fig. 2). A noteworthy point is that these mAbs showed poor cross-reactivity with GyrA subunits from some other Gram-positive bacteria (data not shown), indicating their potential utility in the diagnosis of mycobacterial infections. Similar results have been obtained with polyclonal antibodies raised against *M. tuberculosis* GyrA subunit [17].

Identification of an epitope is a first step towards the elucidation of the molecular mechanisms underlying antigen–antibody interaction. MsGyrA:E9 seems to interact with the N-terminal of GyrA that is likely to be masked in the native enzyme (Figs 3 and 4). Our results suggest that the two inhibitory mAbs, MsGyrA:C3 and MsGyrA:H11 recognize conformation-dependent epitopes (Figs 5 and 6), comprising the structure surrounding amino-acid residues 351 and 415 of GyrA. By sequence alignment with *E. coli* GyrA, this stretch corresponds to amino-acid residues between 340 and 402 of *E. coli* GyrA. Based on the crystal structure of breakage-reunion domain of *E. coli* GyrA [35], these residues form a long helix α14, which is a part of primary dimer interphase. The structure seems to be highly conserved even in yeast topoisomerase II [36], as it forms the second gate for the passage of the T-segment of DNA during catalysis [35]. Thus, the mAb-interacting domain could be forming similar structure in the mycobacterial enzyme. However the differences in this region between the *E. coli* and mycobacterial enzymes could explain the specificity of the interaction of mAbs. Pairwise amino-acid comparison between *E. coli* and *M. smegmatis* in different regions of GyrA revealed a higher degree of differences in this region. Only 44% amino-acid identity was observed in this region compared with 65% identity in the DNA active-site region. A detailed study of the binding of MsGyrA:C3 and MsGyrA:H11 to this region would be required for the better understanding of the mechanistics of the supercoiling reaction performed by mycobacterial gyrase.

As gyrase is both unique and essential to prokaryotes, it has received considerable attention as a molecular target for the generation of new lead molecules. Two major families of DNA gyrase inhibitors have been characterized, the quinolones, which work by interfering with the process of rejoining the double-strand breaks in DNA [3,37] and the coumarins, which affect the ATPase activity of GyrB [38,39]. Furthermore, cyclothialidine, a cyclic peptide, has been characterized to inhibit gyrase activity analogous to the coumarin class of inhibitors [40]. In addition, two proteinaceous poisons against gyrase, microcin B17 [41] and CcdB [42], have been characterized. Two of the mAbs described here form a new class of inhibitors specific for mycobacterial DNA gyrase.

The DNA gyrase supercoiling activity was effectively inhibited by Fab fragments also. Hence it appears that inhibition is caused by direct interaction of the antigen-binding site of these mAbs with GyrA, rather than being a result of a steric effect caused by the Fc regions of the Ig, or by crosslinking of adjacent GyrA molecules. The inhibition of DNA supercoiling was also observed when the pre-formed gyrase–DNA complex was treated with the mAbs (data not shown), implying that these antibodies do not interfere with DNA binding. Based on the interaction pattern and inhibition of the enzyme activity, the mAbs could be grouped into two categories. MsGyrA:C3 and MsGyrA:H11, which interact in a conformation-specific manner and inhibit DNA gyrase supercoiling activity, while MsGyrA:E9 interacts towards the N-terminus of GyrA without affecting enzyme activity.

In summary, the present work highlights the importance of the development of DNA gyrase mAbs for a variety of purposes including issues concerning public health. As the GyrA-specific mAbs described here interact with GyrA from *M. tuberculosis*, *M. bovis*, *M. leprae* and *M. avium*, it opens the avenue to explore their potential value in the diagnosis of mycobacterial infections. It is clear that these mAbs would serve as invaluable tools to study the enzyme in detail to address the role of DNA gyrase in the biology of mycobacteria. In addition to providing information on the structure and function of DNA gyrase, these mAbs are useful for characterizing different complexes and interactions in which DNA gyrase is involved. A point of certain importance is that there is a potential to use the information to develop peptide inhibitors for DNA gyrase as a first step towards lead molecule discovery. The latter point attains considerable significance due to the alarming increase in drug-resistant tuberculosis in recent years.

ACKNOWLEDGEMENTS

We thank A. Maxwell, John Innes Centre, Norwich, UK, for *E. coli* GyrA overexpression clones, P.J. Brennan, Colorado State University, Colorado, USA and V.M. Katoch, Central JALMA Institute for Leprosy, Agra, India for different mycobacterial lysates. We would like to thank S. Shankar for BIAcore studies and D.R. Radha for technical assistance. The Department of Biotechnology, Government of India supported BIAcore facility is acknowledged. This work is supported by a grant from the Department of Biotechnology, Government of India. U.H.M. is the recipient of a Senior Research Fellowship from the Council of Scientific and Industrial Research, Government of India.

REFERENCES

1. Bates, A.D. & Maxwell, A. (1993) *DNA Topology.* Oxford University Press, Oxford.
2. Wang, J.C. (1996) DNA topoisomerases. *Annu. Rev. Biochem.* **65**, 635–692.
3. Reece, R.J. & Maxwell, A. (1991) DNA Gyrase: structure and function. *CRC Crit. Rev. Biochem. Mol. Biol.* **26**, 335–375.
4. Lewis, R.J., Tsai, F.T.E. & Wigley, D.B. (1996) Molecular mechanisms of drug inhibition. *Bioessays* **18**, 661–671.
5. Klevan, L. & Wang, J.C. (1980) DNA gyrase-DNA complex containing 140 bp of DNA and an $\alpha_2\beta_2$ protein core. *Biochemistry* **19**, 5229–5234.
6. Krueger, S., Zaccai, G., Wlodawer, A., Langowski, J., O'Dea, M., Maxwell, A. & Gellert, M. (1990) Neutron light scattering studies of DNA gyrase and its complex with DNA. *J. Mol. Biol.* **211**, 211–220.
7. Bates, A.D., O'Dea, M.H. & Gellert, M. (1996) Energy coupling in *Escherichia coli* DNA gyrase: the relationship between nucleotide binding, strand passage, and DNA supercoiling. *Biochemistry* **6**, 1408–1416.
8. Maxwell, A. (1997) DNA gyrase as a drug target. *Trends Microbiol.* **5**, 102–109.
9. Stahl, D.A. & Urbance, J.W. (1990) The division between fast- and slow-growing species corresponds to natural relationships among mycobacteria. *J. Bacteriol.* **172**, 116–124.
10. Kaufmann, S.H.E. & Van Embden, J.D.A. (1993) Tuberculosis: a neglected disease strikes back. *Trends Microbiol.* **1**, 2–5.
11. Madhusudan, K. & Nagaraja, V. (1995) *Mycobacterium smegmatis* DNA gyrase: cloning and over expression in *Escherichia coli*. *Microbiology* **140**, 3029–3037.
12. Madhusudan, K., Ramesh, V. & Nagaraja, V. (1994) Molecular cloning of *gyrA* and *gyrB* genes of *Mycobacterium tuberculosis*: analysis of nucleotide sequence. *Biochem. Mol. Biol. Int.* **33**, 651–660.
13. Nemoto, T., Sato, N., Iwanari, H., Yamashita, H. & Takagi, T. (1997) Domains structures and immunogenic regions of the 90-kDa heat-shock protein (HSP90). *J. Biol. Chem.* **272**, 26179–26187.
14. Kovalsky, O.I. & Grossman, L. (1998) Accessibility of epitopes on UvrB protein in intermediates generated during incision of UV-irradiated DNA by *Escherichia coli* Uvr(A)BC endonuclease. *J. Biol. Chem.* **273**, 21009–21014.
15. Tournaire, R.B., Demangel, C., Malavaud, B., Vassy, R., Rouyre, S., Kraemer, M., Plouet, J., Derbin, C., Perret, G. & Maize, J.C. (2000) Identification of a peptide blocking vascular endothelial growth factor (VEGF)-mediated angiogenesis. *EMBO J.* **19**, 1525–1533.
16. Nagaraja, V. & Gopinathan, K.P. (1980) Requirement of calcium ions in mycobacteriophage I3 DNA injection and propagation. *Arch. Microbiol.* **124**, 249–254.
17. Manjunatha, U.H., Madhusudan, K., Viswewariah, S.S. & Nagaraja, V. (2000) Structural heterogeneity in DNA gyrases in Gram-positive and Gram-negative bacteria. *Curr. Sci.* **78**, 968–974.
18. Zor, T. & Selinger, Z. (1996) Linearization of Bradford protein assay increases its sensitivity: Theoretical and experimental studies. *Anal. Biochem.* **236**, 302–308.
19. Hallett, P., Grimshaw, A.J., Wigley, D.B. & Maxwell, A. (1990) Cloning of the DNA gyrase genes under tac promoter control: overproduction of the gyrase A and gyrase B proteins. *Gene* **93**, 139–142.
20. Maxwell, A. & Howells, A.J. (1999) *Protocols for DNA Topoisomerases I: DNA Topology and Enzyme Purification* (Bjornsti, M.-A. & Osheroff, N., eds), pp. 135–144. Humana Press. Towata, New Jersey.
21. Viravau, V.R., Triung, Q.C., Moreau, N., Jarlier, V. & Sougakoff, W. (1996) Sequence analysis, purification, and study of inhibition

by 4-quinolones of the DNA gyrase from *mycobacterium smegmatis*. *Antimicrob. Agents Chemother.* **40**, 2054–2061.
22. Shulman, M., Wilde, C.D. & Kohler, G. (1978) A better cell line for making hybridomas secreting specific antibodies. *Nature* **276**, 269–273.
23. Visweswariah, S.S., Karande, A.A. & Adiga, P.R. (1987) Immunological characterization of riboflavin carrier protein using monoclonal antibodies. *Mol. Immunol.* **24**, 969–974.
24. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.
25. Ey, P.L., Prowse, S.J. & Jenkin, C.R. (1978) Isolation of pure IgG_1, IgG_{2a} and IgG_{2b} immunoglobulins from mouse serum using Protein-A Sepharose. *Immunochemistry* **15**, 429–436.
26. Garrett, B.M. & Visweswariah, S.S. (1996) A conformational epitope in the N-terminus of the *Escherichia coli* heat stable enterotoxins is involved in receptor–ligand interactions. *Biochem. Biophys. Acta* **1317**, 149–154.
27. Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
28. Lyn, R.M. & Wang, J.C. (1989) Peptide sequencing and site directed mutagenesis to identify tyrosine-319 as the active site tyrosine of *Escherichia coli* DNA topoisomerase I. *Proteins* **6**, 231–239.
29. Mizuuchi, K., Mizuuchi, M., O'Dea, M.H. & Gellert, M. (1984) Cloning and simplified purification of *Escherichia coli* DNA gyrase A and B proteins. *J. Biol. Chem.* **259**, 9199–9201.
30. Takiff, H.E., Salazar, L., Guerrero, C., Philipp, W., Huang, W.M., Kreisworth, B., Cole, S.T., Jacobs, W.R. Jr & Telenti, A. (1994) Cloning and nucleotide sequence of *Mycobacterium tuberculosis* gyrA and gyrB genes and characterization of quinolone resistance mutations. *Antimicrob. Agents Chemother.* **38**, 773–780.
31. Fsihi, H., Vincent, V. & Cole, S.T. (1996) Homing events in the *gyrA* gene of some mycobacteria. *Proc. Natl Acad. Sci. USA* **93**, 3410–3415.
32. Kirchausen, T., Wang, J.C. & Harrison, S.C. (1985) DNA gyrases and its complexes with DNA: direct observation by electron microscopy. *Cell* **41**, 933–943.
33. Maxwell, A. (1996) Protein gates in DNA topoisomerase II. *Nat. Struct. Biol.* **3**, 109–112.
34. Madhusudan, K. & Nagaraja, V. (1996) Alignment and phylogenetic analysis of type II DNA topoisomerases. *J. Biosci.* **21**, 613–629.
35. Cabral, J.H.M., Jackson, A.P., Smith, C.V., Shiotra, N., Maxwell, A. & Liddington, R. (1997) Crystal structure of the breakage-reunion domain of DNA gyrase. *Nature* **388**, 903–906.
36. Berger, J.M., Gamblin, S.J., Harrison, S.C. & Wang, J.C. (1996) Structure at 2.7 Å resolution of a 92K yeast DNA topoisomerase II fragment. *Nature* **379**, 225–232.
37. Drlica, K. & Franco, R.J. (1988) Inhibitors of DNA topoisomerases. *Biochemistry* **27**, 2253–2259.
38. Hoeksema, H., Johnson, J.L. & Hinman, J.W. (1985) Structural studies on streptonivicin, a new antibiotic. *J. Am. Chem. Soc.* **78**, 6710–6711.
39. Lewis, R.J., Singh, O.M.P., Smith, C.V., Skarzynski, T., Maxwell, A., Wonacott, A.J. & Wigley, D.B. (1996) The nature of inhibition of DNA gyrase by the coumarins and the cyclothialidines revealed by X-ray crystallography. *EMBO J.* **15**, 1412–1420.
40. Nakada, N., Gmunder, H., Hirata, T. & Arisawa, M. (1994) Mechanism of inhibition of DNA gyrase by cyclothialidine, a novel DNA gyrase inhibitor. *Antimicrob. Agents Chemother.* **38**, 1966–1973.
41. Baquero, F., Bouanchauld, D., Martinez-Perez, M.C. & Fernandez, C. (1978) Microcin plasmids a group of extrachromosomal elements coding for low molecular weight antibiotics in *Escherichia coli*. *J. Bacteriol.* **135**, 342–347.
42. Bernard, P. & Couturier, M. (1992) Cell killing by F-plasmid ccdB protein involves poisoning of DNA topoisomerase-II complexes. *J. Mol. Biol.* **226**, 735–745.