



NON-TUBERCULOUS MYCOBACTERIA: GENERAL

Immunological cross-reactivity of mycobacterial topoisomerase I and divergence from other bacteria

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SUMMARY

Mycobacterium smegmatis topoisomerase I exhibits several distinctive characteristics among all topoisomerases. The enzyme is devoid of Zn²⁺ fingers found typically in other bacterial type I topoisomerases and binds DNA in a site-specific manner. Using polyclonal antibodies, we demonstrate the high degree of relatedness of the enzyme across mycobacteria but not other bacteria. This absence of cross-reactivity from other bacteria indicates that mycobacterial topoisomerase I has diverged from *Escherichia coli* and other bacteria. We have investigated further the immunological properties of the enzyme by raising a panel of monoclonal antibodies that recognises different antigenically active regions of the enzyme and binds it with widely varied affinity. Inhibition of a C-terminal domain-specific antibody binding by enzyme-specific and non-specific oligonucleotides suggests the possibility of using these monoclonal antibodies to probe the structure, function and *in vivo* role of the enzyme.

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The enzymes DNA gyrase and topoisomerase I are present in all bacteria, and perform contrasting topological reactions. While DNA gyrase catalyses the introduction of negative supercoils into DNA, the DNA relaxation activity of topoisomerase I removes excessive negative supercoils. The opposing reactions by these essential enzymes are necessary to maintain topological homeostasis within the genome.^{1,2}

A vast majority of bacteria, including *Escherichia coli*, encode four topoisomerases. DNA gyrase and topoisomerase IV are type II enzymes while topoisomerase I and topoisomerase III belong to the type I family. Type I group of enzymes have been further classified into type IA and IB and typically eubacteria encode type IA enzymes. All mycobacteria (with the exception of *Mycobacterium smegmatis*³ and *Mycobacterium avium*⁴) encode only two topoisomerases – DNA gyrase and topoisomerase I in what appears to be the minimal component of enzymes for cell survival. Thus, both DNA gyrase and topoisomerase I would have to share and carry out

all the topological transactions in mycobacteria. Our previous studies have revealed several unexpected properties in *M. smegmatis* topoisomerase I (Msm topo I) that are distinct from other well-characterised type IA enzymes. The enzyme can bind to both single- and double-stranded DNA with high affinity, a property not shared by other type IA topoisomerases. The enzyme recognises DNA in a site-specific fashion and cleaves it at the Strong topoisomerase I site (STS).⁵ The enzyme lacks a Zn²⁺ finger motif, which was shown to be indispensable for function in the case of topoisomerase I from *E. coli* (Eco topo I).^{6,7} These distinct properties of the enzyme, namely site specificity, high-affinity DNA binding and absence of Zn²⁺ fingers, suggest that the Msm topo I has diverged from other typical type IA enzymes to attain its distinct structure and *in vivo* function.

Sequencing of several mycobacterial genomes, including that of *Mycobacterium tuberculosis* and *Mycobacterium leprae*, has made it possible to compare the enzymes and evaluate the extent of their relatedness within the genus and across eubacteria. Using such studies, we show here that anti-Msm topo I polyclonal antibodies recognised only the mycobacterial enzyme and failed to immunologically cross-react with Eco topo I and other bacterial topo I, although Msm topo I and Eco topo I are 46.5% similar. A panel of monoclonal anti-Msm topo I antibodies generated were grouped based on their varied affinities to the holoenzyme and reactivity to the three fragments of the enzyme. We also show that the binding

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of topo I by one of these monoclonals specific to the C-terminal domain (CTD) is inhibited in the presence of the oligonucleotide substrate.

1. Materials and methods

1.1. Bacterial strains and reagents

E. coli (Eco) strains DH10B⁸ and BL21(DE3)⁹ were laboratory strains. ELISA plates and 96-well tissue culture plates were from Becton Dickinson, USA and TPP, Denmark. Complete and incomplete Freund's adjuvant, Iscove's modified Dulbecco's medium (IMDM), foetal bovine serum (FBS), HAT and HT media supplements, anti-mouse IgG–HRP conjugate, bovine serum albumin (BSA) were from Sigma, India. Tetramethyl benzidine (TMB) was from Bangalore Genei, India. The ECL kit was from Amersham Pharmacia Biotech, UK. Sp2/0 cells and hybridoma cells were routinely grown in IMDM containing 10% FBS. Resident peritoneal macrophages obtained by lavage from Balb/c mice were used as feeder cells during fusion and single-cell cloning.

1.2. Preparation of cell lysates from bacterial strains

Cell lysates were prepared from bacterial cultures of *E. coli*, *Shigella sonnei*, *Salmonella typhimurium*, *M. smegmatis*, *Staphylococcus aureus*, *M. tuberculosis* and *Mycobacterium bovis*. The cells were harvested at mid log phase by centrifugation at 10 000 rpm for 10 min at 4 °C and suspended in sonication buffer (20 mM Tris-HCl pH 7.4, 50 mM KCl, 10 mM EDTA and 10% glycerol). The cells were lysed by sonication (Sonic & Materials Inc., Connecticut, USA) and centrifuged at 13 000 rpm for 30 min at 4 °C to collect the cell-free extracts. Protein was estimated by Bradford's method.¹⁰

1.3. Expression and purification of proteins

Msm topo I was over-expressed and purified from *E. coli* BL21(DE3) cells transformed with the expression plasmid vector pPVN123, as mentioned previously.¹¹ The protein was purified to near homogeneity by several steps, including ammonium sulphate precipitation, Hi-Trap heparin sepharose chromatography followed by Hi-Trap SP sepharose column (Figure 1A). Truncated topoisomerase I fragments corresponding to NTD1 (aa1 to aa240), NTD2 (aa241 to aa612) and CTD (aa613 to aa938) were individually cloned into pBS (KS+) vectors before being introduced into pRSETA expression vectors for over-expression. For purification of topo I fragments, transformed and IPTG-induced *E. coli* cells were sonicated and the cell lysate was centrifuged at 20 000 g. The supernatant was loaded onto a preparative 10% SDS-PAGE and the relevant band corresponding to the fragment was electro-eluted and dialysed against 10 mM phosphate buffer, pH 7.4.

1.4. Generation of monoclonal antibodies against *M. smegmatis* topoisomerase I

Balb/c female mice about 6–8 weeks old were immunised subcutaneously with 50 µg of protein in a total volume of 100 µl after emulsification in equal volume of complete Freund's adjuvant (CFA). Two booster doses of 50 µg each were given at 2-week intervals in incomplete Freund's adjuvant (IFA). Three days prior to fusion, the mice were immunised with 50 µg protein in phosphate-buffered saline (PBS) intraperitoneally. On the day of fusion, spleen cells were isolated and fused with Sp2/0 myeloma cells,^{12–14} and hybrids were selected in HAT medium. Monoclonality was established by performing limiting dilution at 0.1 cell per well at least twice after detecting antibody-secreting clones by ELISA.

1.5. ELISA and monoclonal antibody titres

A total of 100 ng of Msm topo I was coated in PBS at 37 °C for 2 h. After blocking for 1 h with 0.2% BSA at 37 °C, 100 µl of culture supernatants, diluted in 0.2% BSA, as mentioned in the figure legends, were added and incubated for 30 min at 37 °C. Then 100 µl of anti-mouse IgG–HRP (1:5000 dilution) was added and incubated for another 30 min. Colour was developed by adding 100 µl of TMB, incubated at room temperature in dark for 5 min and the reaction was stopped by adding 100 µl of 3 N H₂SO₄. The optical density (OD) readings were measured at 450 nm (Spectramax Softmax pro ELISA reader, USA). All washings were carried out with PBST (PBS with 0.05% Tween-20) thrice at each step. The OD readings were plotted using the GraphPad Prism version 4 software after subtracting baseline values. The dilution of the supernatant, at which 50% of maximum OD₄₅₀ value was obtained, was considered the 50% saturation titre of that particular supernatant. The OD₄₅₀ value obtained at saturation was defined as the maximum and the values obtained were analysed using the non-linear Boltzman sigmoidal fit, with an *r*² value of 0.98 or greater. All titration curves and competitive ELISA assays were done with the same aliquot of supernatant collected from different clones that had been cloned to monoclonality by limiting dilution.

When using oligonucleotides, plates were coated and blocked as mentioned. The PBS-washed wells were then incubated with a mixture of monoclonal antibody and 100 ng of a 32-mer oligonucleotide containing the STS (5'-CAGTGAGCGAGCTTCC GCTTGA CATCCAATA-3') in 100 mM potassium phosphate (pH 7.0) buffer in a total volume of 100 µl for 30 min at 37 °C. Monoclonal supernatants were used at 50% saturation titre. The plates were then incubated with IgG–HRP conjugate and colour developed as mentioned above. A 32-mer oligonucleotide complementary to the STS-oligo sequence was used as the non-specific control. In experiments involving prior addition of oligonucleotide or 1A6C11, the STS-oligo was added first to topo I-coated wells and incubated for 30 min at 37 °C before 1A6C11 addition. Conversely, topo I-coated wells were incubated with 1A6C11 for 30 min at 37 °C before addition of STS-oligo. In both the cases, further incubation was carried out at 30 min at 37 °C before addition of the IgG–HRP conjugate.

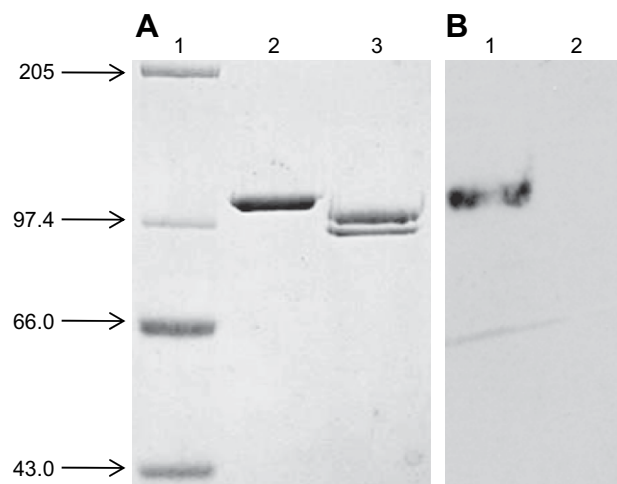


Figure 1. Cross-reactivity of purified Msm topo I and Eco topo I. Panel A shows the Coomassie blue staining of purified (1 µg) *M. smegmatis* topo I (lane 2) and *E. coli* topo I (lane 3) subjected to SDS-PAGE. Molecular weight markers are included in lane 1. Panel B shows immunoblotting of *M. smegmatis* topo I (lane 1) and *E. coli* topo I (lane 2) using 1:10,000 diluted mouse antisera against *M. smegmatis* topo I.

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