Reduction in DNA topoisomerase I level affects growth, phenotype and nucleoid architecture of *Mycobacterium smegmatis*

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The steady-state negative supercoiling of eubacterial genomes is maintained by the action of DNA topoisomerases. Topoisomerase distribution varies in different species of mycobacteria. While *Mycobacterium tuberculosis* (*Mtb*) contains a single type I (TopoI) and a single type II (Gyrase) enzyme, *Mycobacterium smegmatis* (*Msm*) and other members harbour additional relaxases. TopoI is essential for *Mtb* survival. However, the necessity of TopoI or other relaxases in *Msm* has not been investigated. To recognize the importance of TopoI for growth, physiology and gene expression of *Msm*, we have developed a conditional knock-down strain of TopoI in *Msm*. The TopoI-depleted strain exhibited extremely slow growth and drastic changes in phenotypic characteristics. The cessation of growth indicates the essential requirement of the enzyme for the organism in spite of having additional DNA relaxation enzymes in the cell. Notably, the imbalance in TopoI level led to the altered expression of topology modulatory proteins, resulting in a diffused nucleoid architecture. Proteomic and transcript analysis of the mutant indicated reduced expression of the genes involved in central metabolic pathways and core DNA transaction processes. RNA polymerase (RNAP) distribution on the transcription units was affected in the TopoI-depleted cells, suggesting global alteration in transcription. The study thus highlights the essential requirement of TopoI in the maintenance of cellular phenotype, growth characteristics and gene expression in mycobacteria. A decrease in TopoI level led to altered RNAP occupancy and impaired transcription elongation, causing severe downstream effects.

**INTRODUCTION**

Genome compaction is necessary to accommodate it within the small intracellular compartment of the bacterial cell. Bacterial DNA compaction is facilitated by the introduction of negative supercoiling activity of DNA topoisomerases (Deng et al., 2005; Saier, 2008; Wang, 2002). Negative supercoiling enhances the free energy of the DNA, which facilitates melting of the promoter elements during transcription initiation. Potentially, alteration in the genome supercoiling/DNA topology can affect transcription (Travers & Muskhelishvili, 2005). Molecular and genetic evidence suggests that several environmental cues modulate the supercoiling/DNA topology of the bacterial genome (Drlica, 1992), which consequently influences gene expression (Dorman, 1991). Anaerobic growth, osmolarity and temperature have been shown to affect supercoiling of the DNA (Dorman et al., 1988; Goldstein & Drlica, 1984; Higgins et al., 1988). Genome supercoiling, in turn, appears to sense, interpret and co-ordinate the expression of vast sets of genes contributing to adaptation to various environments (Dorman, 1991, 2006).

Supercoiling of the bacterial genome is maintained by the countervailing activities of the DNA gyrase and Topol (DiNardo et al., 1982; Pruss et al., 1982; Wang, 1985). Topol relaxes excess negative supercoiling accumulated due to the action of replication and transcription machineries while DNA gyrase introduces negative supercoiling in an ATP-dependent manner to maintain optimal negative supercoiling (Champoux, 2001). The metabolic state or environmental cues alter the [ATP]/[ADP] ratio in the cell (Hsieh et al., 1991; Westerhoff et al., 1988), which in turn influences DNA gyrase activity resulting in a change in the superhelical density of the genome (Drlica, 1992; Hsieh et al., 1991). Any alteration or imbalance in topoisomerase expression and/or activity inside the cell can modulate gene expression. Indeed, mutations in the genes or the actions of inhibitors on these
major topology modulatory enzymes lead to various pleiotropic effects on the cellular processes of various bacteria. For instance, in a topoisomerase I (TopoI)-deficient mutant of *Salmonella Typhimurium*, osmotic induction of the invA gene is affected, leading to diminished invasiveness (Galán & Curtiss, 1990). The transcription of the invasive genes of the organism is also perturbed by the inhibition of DNA gyrase (Dorman & Ni Bhriain, 1993). In *Escherichia coli*, *Streptococcus pneumoniae* and *Haemophilus influenzae*, the total transcriptome was altered upon DNA gyrase inhibition, suggesting a global regulatory role of genome supercoiling (Ferrándiz et al., 2010; Gmuender et al., 2001; Peter et al., 2004). Furthermore, proteomic study with a TopoI and DNA gyrase mutant revealed an altered proteome of *E. coli* (Steck et al., 1993). From these studies, it is evident that DNA topoisomerases play a crucial role in the physiology and gene expression of eubacteria (Hatfield & Benham, 2002).

The genus *Mycobacterium* comprises a variety of organisms including various slow-growing pathogens such as *Mycobacterium tuberculosis* (*Mtb*), *Mycobacterium leprae*, *Mycobacterium marinum* and *Mycobacterium abscessus* as well as the well-studied, non-pathogenic, fast-growing *Mycobacterium smegmatis* (*Msm*). In contrast to the slow-growing *Mtb*, which contains a single TopoI for genome relaxation, *Msm* has an unusual type II enzyme, TopoNM, and an uncharacterized type IB topoisomerase apart from TopoI to carry out DNA relaxation (Jain & Nagaraja, 2005). While the indispensability of TopoI in *Mtb* has been demonstrated recently (Ahmed et al., 2014), the presence of surplus DNA relaxation enzymes raises the question about the importance of TopoI in *Msm* for growth and gene regulation. Having additional DNA relaxation enzymes in the *Msm* genome would suggest the dispensability of TopoI as the other two enzymes could cope with the cellular DNA relaxation burden. Furthermore, the role of topoisomerases and genome supercoiling in the physiology of mycobacteria has yet to be explored. Several studies have unravelled the distinctive features of mycobacterial TopoI, such as site-specific DNA binding, high processivity and the ability to bind both single- and double-stranded DNA (Bhaduri et al., 1998a, b). These distinct properties of *Msm* TopoI may have vital functions and regulatory roles. Thus, to monitor the influence of alterations in TopoI level on mycobacterial physiology and growth, a conditional knock-down strain of TopoI was generated in *Msm* using the TetR-pip-based system (Boldrin et al., 2010). The mutant with reduced expression of TopoI exhibited a severe growth defect, altered nucleoid architecture and phenotypic variations, indicating the global regulatory role of TopoI in the biology of *Msm*.

**METHODS**

**Bacterial strains, growth media and transformation conditions.** The following bacterial strains were used: *E. coli* DH10B (laboratory stock) and *Msm* mcΔ155 (laboratory stock). *E. coli* strains were grown at 37°C in Luria–Bertani (LB) broth or on LB agar plates. Mycobacterial strains were grown at 37°C in Middlebrook 7H9 broth (Difco) or on 7H10 agar plates (Difco), supplemented with 0.2% glycerol and 0.05% Tween 80. For growth of MsPptrtopoI (a *topoI* conditional mutant), the medium was supplemented with 10% ADC (albumin, glucose and NaCl) and cultures were grown at 30°C. Antibiotics were added to the media at the following concentrations: streptomycin, 20 μg ml⁻¹; kanamycin, 50 μg ml⁻¹; hygromycin, 150 μg ml⁻¹ (*E. coli*) or 50 μg ml⁻¹ (*Msm*). Anhydroretetracycline (ATc) (Sigma-Aldrich) was added, when required, at final concentrations from 10 to 200 ng ml⁻¹.

**Construction of conditional mutant of TopoI in *Msm*.** The first 750 bp fragment of *Msm* *topoI* was cloned downstream of the ptr promoter region in the suicide plasmid pFRA50 to obtain pFRA50-MstopoI. To replace the promoters of *topoI* with the Pip-controlled promoter Pptr, *Msm* cells were electroporated with 2 μg of plasmid (pFRA50-MstopoI). Recombinant colonies were selected on 7H10 agar plates containing hygromycin (50 μg ml⁻¹). Integration of the plasmid via insertional duplication was confirmed by PCR. The resulting recombinant strain was transformed with the integrative plasmid pFRA42B (containing the TetR/Pip system) to obtain an *Msm* *topoI* conditional mutant (MsPptrtopoI). For complementation analysis, TopoI over expression constructs were generated in the pMIND vector system (Blokoel et al., 2005). The MstopoI gene was amplified from pPVN123 (Jain & Nagaraja, 2006). The PCR products were digested with *Ndel* and EcoRV and cloned into the *Ndel* and EcoRV linearized pMIND vector. Clones were confirmed by double digestion with *Ndel* and BamHI digestion, and the expression of MstopoI in *Msm* cells was monitored by immunoblotting.

**Growth analysis of MsPptrtopoI.** Colonies of MsPptrtopoI were grown on 7H10 agar at 30°C. The cultures of the conditional mutant were grown at 30°C in Middlebrook 7H9 broth supplemented with 0.2% glycerol, 0.05% Tween 80 and 10% ADC. Wherever needed, MsPptrtopoI and WT cultures were treated with 10–200 ng ATc ml⁻¹ for the indicated periods of time. For measuring the growth of *Msm* and the mutant, exponential phase cultures (150 μg ml⁻¹) were taken and diluted in 7H9 media to an OD₆₀₀ 0.1. Aliquots of concentrated cells (at densities of 5 x 10⁸ c.f.u ml⁻¹) were decoted in 7H9 broth (Difco) or on 7H10 agar plates (Difco) and were grown at 37°C for 10–200 ng ml⁻¹. The resulting recombinant strain was transformed with the integrative plasmid pFRA42B (containing the TetR/Pip system) to obtain an *Msm* *topoI* conditional mutant (MsPptrtopoI). For complementation analysis, TopoI over expression constructs were generated in the pMIND vector system (Blokoel et al., 2005). The MstopoI gene was amplified from pPVN123 (Jain & Nagaraja, 2006). The PCR products were digested with *Ndel* and EcoRV and cloned into the *Ndel* and EcoRV linearized pMIND vector. Clones were confirmed by double digestion with *Ndel* and BamHI digestion, and the expression of MstopoI in *Msm* cells was monitored by immunoblotting.

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**Immunoblot analysis.** Proteins were separated on 8% SDS-PAGE and transferred to PVDF membranes. Membranes were incubated in PBS blocking buffer (10 mM sodium phosphate, pH 7.5, 150 mM NaCl, 0.05% Tween 20) with 2% (w/v) BSA for 2 h prior to incubation with primary antibodies diluted (1:20,000) in PBS with 2% BSA for 2 h. Membranes were washed in PBST (0.2% Tween 20) three times, and incubated with horseradish peroxidase-conjugated goat polyclonal anti-mouse IgG or goat polyclonal anti-rabbit IgG secondary antibodies (GE Amersham) (dilution 1:20 000) for 2 h followed by washing three times with PBST. Protein bands were visualized using chemiluminescent substrates (Millipore).

**Scanning electron microscopy and fluorescence (DAPI) microscopy.** Culture aliquots of 5 ml were concentrated by centrifugation (5000 g) before suspending in fresh Middlebrook 7H9 medium. Aliquots of concentrated cells (at densities of 5 x 10⁸ c.f.u. ml⁻¹) were placed on poly-L-lysine-coated coverslips in 24-well tissue culture plates. Bacteria were allowed to settle for 30 min before gently decanting and adding 1 ml solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) with 0.2 M sucrose. Samples were treated with 2% OsO₄ in 0.1 M sodium cacodylate buffer for 2 h at room temperature. A series of sequential ethanol dehydrations were performed for 10 min each (30, 50, 70, 95 and 100%) before drying the samples under vacuum.
Plasmid isolation and chloroquine/agarose gel electrophoresis. Exponential phase cultures of WT and mutant (MsPptrtopoI) harbouring the pMV261 plasmid were treated with glycine (1%, w/v) for 3 h followed by harvesting of the cells. The plasmids were isolated using the Qiagen Plasmid Midi kit as per the manufacturer’s instructions. Then, 2 µg of the purified plasmids was subjected to chloroquine/agarose gel (1.2%, w/v) electrophoresis using 1 X TAE buffer containing 2.5 µg chloroquine diphosphate ml⁻¹ (Sigma-Aldrich). Electrophoresis was carried out at room temperature by applying a voltage gradient of 2 V cm⁻¹ for 16 h. Following electrophoresis, the gels were washed with double-distilled water and topoisomerasers were visualized by staining with ethidium bromide (EtBr, 0.5 µg ml⁻¹).

Biofilm, pellicle and motility assays. Biofilm formation was assessed as described previously (Ghosh et al., 2013; Recht et al., 2000). Briefly, 100 µl of 7H9 medium supplemented with 0.5% Casamino acids (casein hydrolysate) was used per well in a 96-well PVC microtitre dish. The medium was inoculated with exponential phase cells and microtitre dishes were incubated at room temperature for 48 and 96 h for the WT and mutant, respectively (to an A₅₆₀ of 0.5). The wells were rinsed twice with water, and 120 µl of a 1% cell-staining solution of crystal violet (CV) was added. Plates were incubated at room temperature for 30 min, rinsed with water three times and scored for CV staining. Quantification of biofilm formation was performed by extracting the biofilm-associated CV with 100% ethanol for 1 h and measuring the optical density at 570 nm. Pellicle formation was monitored by growing standing cultures of mycobacteria in Middlebrook 7H9 medium without Tween 80 at 37°C.

Motility assays were carried out as described previously (Martı́nez et al., 1999). Briefly, cells were cultured in 7H9 medium to mid-exponential phase (A₅₆₀ of 0.4–0.6) before spotting 2 µl aliquots onto motility medium consisting of 7H9 supplemented with 0.5% Casamino acids and 0.2% glycerol, solidified with low-melting-point agarose (0.4%, w/v). The inoculated plates were incubated for 24–96 h at 30°C in plastic bags containing moistened tissue paper to ensure that the bacteria grew under humidified conditions.

Purification and analysis of glycopeptidolipids (GPLs). The GPLs from Msm cells were purified as described by Khoo et al. (1996). Briefly, lipids were extracted by treating 5 g (dry weight) of cells at exponential phase with chloroform/methanol (2:1) for 24 h at room temperature. The organic supernatant was discarded and the organic layer containing the lipids was dissolved in chloroform/methanol/water (2:1:1). The aqueous layer was discarded and the organic layer containing the lipids was concentrated. The de-acetylated lipids were spotted on to a silica-coated TLC plate (Merck) and mobilized with chloroform/methanol (9:1). The sugar-containing lipids were visualized by spraying the plate with 10% H₂SO₄ in 1% 1-naphthol (in ethanol) followed by heating at 120°C for 10 min.

RNA extraction and quantitative real-time PCR (qPCR). RNA was extracted from Msm cells using a Qiagen RNeasy kit according to the manufacturer’s protocol. From the total RNA, cDNAs were synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems). cDNA generated with random primers was used for qPCR, with SYBR green as the indicator dye. Expression of the genes was quantified after normalization of RNA levels to expression of the sigA gene. The qPCR cycling conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 57°C for 30 s and 72°C for 20 s.

EtBr uptake assay. The exponentially grown cells were diluted to an A₅₆₀ of 0.4 and treated with EtBr (0.5 µg ml⁻¹). The EtBr-loaded cells were centrifuged at 4000 rpm for 3 min and resuspended in EtBr-free PBS containing 0.4% glucose. After adjusting the OD₅₆₀ to 0.4, aliquots of 100 µl were transferred to a 1.4 ml cuvette. Fluorescence was measured in a fluorimeter (Hitachi) using the 290 nm band-pass and the 580 nm high-pass filter as the excitation and detection wavelengths, respectively.

2D gel electrophoresis and MS (2D-MS). Msm (WT) and MsPptrtopoI cells were grown in 7H9 medium at 30°C to an A₅₆₀ of 0.6. The cells were harvested, washed twice with PBS and resuspended in lysis buffer (10 mM HEPES, pH 8.0, 7% urea, 4% CHAPS, 2 M thiourea, 10 mM DTT), followed by sonication. The lysate was processed with a ReadyPrep 2-D Cleanup kit (Bio-Rad). Immobilized pH gradient strips, pH 3–10 (Bio-Rad; 17 cm), were rehydrated with 300 µl of total protein lysate. IEF was carried out on a Protean i12 IEF Cell (Bio-Rad) using the following protocol: (i) 0–500 V linear for 2 h, (ii) 250 V rapid for 2 h, (iii) 250–6000 V linear for 3 h and (iv) 6000 V constant to 50 kV.h. After equilibration, the strips were loaded and resolved on 12% SDS-PAGE gels. The gels were stained with a ProteoSilver kit (Sigma-Aldrich), and the spots which differed in intensity between samples were identified by MS.

Chromatin immunoprecipitation (ChIP) and real-time PCR. ChIP with exponentially grown Msm cultures was carried out as described previously (Uplekar et al., 2013). Briefly, formaldehyde cross-linked cells were sonicated to shear the DNA using a Diagenode Bioruptor device. The fragmented DNA was immune-precipitated by using the anti-RpoB antibody and purified. The resulting ChIP-DNA was subjected to real-time PCR analysis to determine the enrichment of the target DNA in the IP sample over the mock-IP sample. The results were expressed as the enrichment of RNA polymerase (RNAP) on target DNA in the mutant normalized to the enrichment value of the WT samples.

RESULTS

Growth analysis of the conditional knock-down strain

The top1 knock-down strain of Msm was generated by replacing the native promoter of top1 with the ptr promoter as described in Methods. Successful replacement of the promoter was confirmed by PCR analysis (data not shown) and the level of Topol in the knock-down strain was monitored by immunoblotting. Notably, expression of Topol in the mutant strain was 1.5-fold lower than the WT strain, even in the absence of ATc (Fig. 1a). Addition of ATc led to a further 2.5-fold reduction in the level of the protein. Thus, the MsPptrtopoI strain is a knock-down strain in which the level of Topol is intrinsically lower and could be further reduced by the addition of ATc.
Growth analysis indicated that TopoI mutant cells took 8 days to appear as colonies on 7H10 agar, as compared with 4 days for the WT. The slower growth of the mutant could be attributed to the reduced level of TopoI (Fig. 1a). Notably, the mutant failed to grow in the Middlebrook 7H9 medium used for growth of WT cells, and growth resumed only upon supplementation of 10% ADC in the medium (Fig. 1b). Additionally, the cultures showed temperature sensitivity and exhibited very slow growth at 37°C; mutant cultures reached the exponential phase after 96 h (data not shown). Growth was improved by lowering the temperature to 30°C. All the cultures were therefore grown in the presence of 10% ADC at 30°C. From these observations, it is apparent that an optimal TopoI expression is required for normal growth of Msm. To further evaluate the necessity of TopoI for mycobacterial growth, the cultures were grown in the presence of different concentrations of ATc (Fig. 1c). The analysis indicated a prolonged lag phase of the TopoI-depleted cells compared with the WT. With an increase in ATc concentrations, growth was severely compromised. Importantly, growth of the mutant was rescued upon complementation with the plasmid copy of Mtb topol (MttopoI) or Msm topol (MstopoI) (Fig. 1d), confirming that the reduced TopoI activity inside the cell resulted in reduced growth. The rescue of Msm cells depleted of TopoI by complementing with MttopoI-expressing plasmid suggests similar in vivo roles of the enzymes, in accordance with their similar biochemical properties (Godbole et al., 2012). The requirement of additional growth supplements for growth of the mutant indicated that cellular metabolism is affected in this strain.

**Colony morphology and surface phenotypes of the mutant**

The severely compromised growth rate, requirement of nutrient supplements and low temperature for growth of the TopoI mutant suggested an altered metabolism that could affect colony and cell morphology. Indeed, the TopoI-depleted strain acquired a rough colony morphology and cells tended to cluster towards the centre of the colony while the WT strain maintained the smooth colony morphology (Fig. 2a). Furthermore, scanning electron microscopy of the conditional mutant strain revealed an irregular cell structure and bulb-like protrusions on the cell surface indicating a defective cell surface (data not shown). The change in colony morphology and cell surface of the mutant cells suggested modification of other cell surface-related phenotypes. Because of a high lipid content, mycobacteria grown in the absence of detergents form a pellicle in standing cultures (Etienne et al., 2002). In contrast to the WT strain, the mutant was unable to form a pellicle (Fig. 2d). A pellicle is the biofilm formed at the air–water interface (Branda et al., 2005; Solano et al., 2002) and a relationship exists between the ability to form a pellicle and...
biofilm. To better assess and quantify the potential of the mutant to form a biofilm, cultures were grown in polystyrene plates. Biofilm-forming ability of the mutant was significantly compromised (about sixfold) as compared with the WT (Fig. 2c).

Another surface-related phenotype in *Msm* is sliding motility, which is restricted to smooth colonies (Agustí et al., 2008). As the mutant acquires rough and dried colony morphology, sliding motility was evaluated. The mutant was drastically affected in sliding motility and did not show the typical halo formation that is characteristic of sliding motility (Fig. 2b). Notably, the surface phenotypes were rescued by the ectopic expression of MstopoI (Fig. 2). These results indicate that the reduced level of TopoI in *Msm* confers pleiotropic effects resulting in altered surface properties and cell phenotypes of the mutant strain.

**Lipid profile of Topol-depleted Msm cells**

Colony morphology, sliding motility and pellicle and biofilm formation are influenced by the lipid constituents of the *Msm* cell envelope (Etienne et al., 2005). The role of GPLs in the aforementioned phenotypes is well established (Etienne et al., 2002; Recht & Kolter, 2001). To evaluate whether altered surface properties of the Topol mutant could be due to an altered lipid profile, the GPLs from the WT, Topol knock-down and MstopoI complemented strain were isolated. An equal amount of dry cell mass was taken, and GPLs were isolated according to established protocols (Khoo et al., 1996) and then subjected to TLC. Analysis of the TLC data indicated the presence of characteristic spots of the polar GPLs in the WT strain (Patterson et al., 2000) that were significantly reduced in the MsPptrtopoI strain (Fig. 3a). The influence of Topol deprivation on GPL biosynthesis was further evaluated by monitoring the expression of the *mps* and *mmpl4b* genes, which are known to be involved in GPL biosynthesis (Billman-Jacobe et al., 1999). Transcriptional analysis suggested a significantly reduced expression of *mps* and *mmpl4b* in the Topol-depleted cells as compared with the WT (Fig. 3b). The data indicate that the reduction in Topol level affects transcription of the genes involved in

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**Fig. 2.** Altered phenotype of MsPptrtopoI. (a) Colony morphology was demonstrated by growing the colonies on 7H9 agar for 8 days. (b) Sliding motility was determined on 0.4% agar. (c) Biofilm formation: the quantification of biofilm formation ability was carried out using CV staining. (d) Pellicle formation was seen in the static culture without Tween 80.
the lipid metabolic pathway, resulting in altered surface properties of mycobacteria.

Topol-depleted strain acquires phenotypic drug resistance

Altered surface properties and GPL levels of the mutant strain may influence cell permeability, which can affect drug intake (Etienne et al., 2002) and thus alter the susceptibility of the cells to such compounds. Rifampicin and isoniazid, widely used anti-tuberculosis drugs, were tested for their effect on survival of the mutant. The MsPptrtopoI mutant strain exhibited reduced sensitivity to the drugs in comparison with the WT (Fig. 4a). The increased survival of the topol knock-down strain could be due to the low metabolic activity or altered permeability of the cell. The altered cell envelope could have a pronounced effect on cell wall permeability (Etienne et al., 2002). Cell permeability was determined by scoring EtBr uptake by the cells (Rodrigues et al., 2011). The mutant was compromised in EtBr uptake, indicating decreased cell permeability compared with the WT (Fig. 4b), which may contribute to phenotypic drug resistance in the mutant.

Reduction in Topol leads to nucleoid de-compaction and altered supercoiling

In a bacterial cell, DNA topology is regulated by the combined action of topoisomerases and nucleoid-associated proteins (NAPs) (Dillon & Dorman, 2010; Espeli & Marians, 2004). These two different kinds of topology modulators function in concert to organize the bacterial chromosome. To explore the effect of Topol deprivation on nucleoid architecture, the nucleoid was examined by DAPI fluorescence microscopy. The nucleoid of Topol-deprived cells was in a de-condensed state across the bacterial cell, while the nucleoid of WT cells remained condensed (Fig. 5a). As NAPs and topology modulatory proteins are responsible for the maintenance of nucleoid architecture, altered structure of the nucleoid might be a consequence of perturbation in expression levels of these proteins. To test this, the expression level of the major DNA topology regulators was monitored. In response to the reduction in Topol level, expression of DNA gyrase was also reduced, suggesting that co-ordinated expression of topoisomerases is required to maintain topological homeostasis (Fig. 5b). The expression of TopoNM, an atypical type II topoisomerase found in Msn, was also reduced in
the topol knock-down strain (22-fold compared with the WT). Moreover, transcript levels of the major NAPs (HU and Lsr2) were also reduced in Topol-depleted cells (Fig. 5c). It is apparent that the imbalance in Topol levels brings about a change in nucleoid architecture as a consequence of variation in the levels of other important topology modulators.

Reduction in Topol expression affects the supercoiling status of the genome (Pruss et al., 1982). To investigate the level of DNA supercoiling in Topol-depleted Msm cells, plasmid supercoiling was directly measured by isolating a reporter plasmid (pMV261) and was analysed by chloroquine/agarose gel electrophoresis to resolve the various topoisomers. The increased accumulation of hyper-negative supercoiled plasmids in the Topol-depleted cells compared with plasmids isolated from the WT cells (Fig. 5d) indicated that the reduction in the level of Topol confers higher levels of negative supercoiling.

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**Fig. 5.** Perturbation of DNA topology modulators and nucleoid decompaction. (a) Exponential phase cultures of WT and conditional mutant were treated with ATc (100 ng ml$^{-1}$), stained with DAPI and visualized by fluorescence microscopy. (b) Immunoblot analysis of Topol and GyrB expression in the conditional mutant. (c) Transcript expression analysis of major topology modulators under Topol-deprived conditions in the mutant. (d) pMV261 plasmids isolated from exponential phase mycobacterial cells were subjected to chloroquine/agarose gel electrophoresis.
TopoI deprivation affects the proteome

From the results presented, it is evident that the reduced level of TopoI and hence perturbation of DNA topology results in various pleotropic effects affecting the physiology and genome organization of \textit{Msm}. These variations in the properties of the mutant cells indicate global changes in gene expression. Previous studies in \textit{E. coli} with a TopoI mutant indicated that the increase in steady-state DNA supercoiling levels resulted in an altered abundance of various proteins (Steck \textit{et al.}, 1993). To investigate the consequences of TopoI deprivation on global protein expression, 2D gel electrophoresis was carried out. Comparison of the proteome of the WT and TopoI-deprived cells demonstrated that multiple proteins were differentially expressed between the two strains (Fig. 6a, b). Few of the differentially expressed proteins were identified by peptide mass fingerprinting–MS. These proteins are involved in diverse cellular pathways such as metabolic processes (FabG and aldolase), transport (SseC – a component of secretion system III) and chaperonin (GroS) functions. Variation in the level of expression of these genes was confirmed by transcript analysis (Fig. 6c). Together, it appears that perturbation of steady-state supercoiling in \textit{Msm} by reduction in TopoI levels could lead to global changes in gene expression. These results complement previous observations in \textit{E. coli} in which mutation in \textit{topoI} affected expression of various genes and operons (Steck \textit{et al.}, 1993; Sternglanz \textit{et al.}, 1981).

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig6.png}
\caption{Identification of differentially expressed proteins in TopoI-depleted \textit{Msm}. Silver-stained 2D electrophoresis gels of (a) WT and (b) MsPptrtopoI. IEF of cell lysates was carried out on 17 cm immobilized pH gradient strips at pH 3–10, and 14\% SDS-PAGE was used for resolution in the second dimension. Arrows indicate the differentially expressed protein spots in the two strains subjected to MS. (c) Real-time PCR validation of the genes identified by 2D MS. Change in gene expression is represented as a ratio of transcripts detected in MsPptrtopoI and the transcripts detected in WT.}
\end{figure}
Topol deprivation affects RNAP distribution across the transcription units (TUs)

The aforementioned analysis of the Topol mutant indicates global changes in cell physiology and gene expression. Topol participates during the transcription process to remove excess negative supercoils generated as a result of the advancement of RNAP along the template DNA (Broccoli et al., 2004; Massé & Drolet, 1999; Pruss & Drlica, 1986). Hence, the reduction in level of Topol would result in reduced relaxation activity, resulting in the accumulation of torsional stress across the genome and TUs, which may affect the dynamics and distribution of RNAP. To evaluate the influence of Topol depletion on RNAP distribution, we analysed the occupancy of RNAP across the TUs of the genes affected in expression. The occupancy of RNAP was reduced significantly on the ORF of the genes tested (Fig. 7a), suggesting a reduction in RNAP molecules in the elongation phase. To gain further insight into the distribution of RNAP on the TUs, the ratio (travelling ratio) of RNAP occupancy on the gene promoter (gene head) and ORF (gene body) was determined (Reppas et al., 2006; Wade & Struhl, 2008). The increased travelling ratio values indicated the accumulation of RNAP on the gene head and reduction in the elongating RNAP, demonstrating aberrant elongation in the Topol-depleted state (Fig. 7b). Thus, it appears that reduction in Topol levels affects the DNA topology of the genome, leading to inefficient transcription of several genes conferring various pleiotropic effects.

**DISCUSSION**

A number of species of mycobacteria, including *Mtb*, possess a single type I topoisomerase and a DNA gyrase to achieve topological inter-conversions in the cell. In contrast, a large number of actinomycetes, including *Msm*, have additional topoisomerases. In addition to an extra orphan GyrB (Jain & Nagaraja, 2002) and an unusual type II relaxase termed TopoNM (Jain & Nagaraja, 2005), the *Msm* genome encodes a type IB (MSMEG _1784) topoisomerase (http://mycobrowser.epfl.ch/smegmalist.html) similar to that found in poxviruses. Such a type IB enzyme is known to possess DNA relaxation activity in *Deinococcus radiodurans* (Krogh & Shuman, 2002). The presence of additional topoisomerases in the genome suggests their role in assisting the principal enzyme in DNA relaxation and maintaining topological homeostasis. This, in turn, may imply the functional redundancy of DNA relaxation enzymes in *Msm*, other fast-growing mycobacteria and members of the genus *Streptomycetes*. Thus, it was important to investigate whether Topol is absolutely essential for *Msm* survival or if the cells could cope with the ‘back-up’ relaxation machinery when Topol levels were depleted. The results presented in this study demonstrate that Topol in *Msm* is essential for cell growth and proliferation. The variation in levels of Topol conferred altered colony morphology and phenotypic characteristics. Additionally, reduced levels of the enzyme resulted in altered expression of other topology modulatory proteins, and the consequent diffused nucleoid revealed the importance of Topol in the maintenance of nucleoid architecture. In contrast to the Topol mutant of *Msm* which acquires phenotypic drug resistance, the *Mtb* Topol mutant did not show drug resistance (Ahmed et al., 2014). Instead, the *Mtb* strain exhibited enhanced susceptibility to novobiocin and isoniazid upon depletion of Topol with ATc (Ahmed et al., 2014). These seemingly opposing results are actually due to the different levels of Topol in the two species. Analysis of levels of Topol in the mutant strains of both organisms (in the absence of ATc) indicated reduced levels of Topol in the *Msm* mutant compared with the WT strain, whereas in the *Mtb* mutant levels were higher than in the WT. These results suggest variation in the expression levels of the enzyme in the two species due to differences in their respective promoter activity. Thus, the alteration in levels of Topol or supercoiling alterations in different organisms may have different effects, as illustrated previously in *Salmonella* and...
E. coli (Cameron et al., 2011). Notably, two clinical strains of Salmonella having different mutations in gyrase genes exhibited diverse supercoiling and phenotypic properties, suggesting that variations in levels of topoisomerase activities can influence the physiology of the organism differentially (Webber et al., 2014).

TopoI-depleted Msm exhibited a growth defect and pleotropic phenotypes (shown schematically in Fig. 8). The mutant acquired altered physiology, metabolism and drug resistance due to alteration in levels of TopoI or consequent altered genome supercoiling influencing diverse pathways leading to global changes. The importance of topoisomerasers in the maintenance of genome supercoiling and thus fine-tuning of the expression of various genes involved in adaptation, stress and virulence has been studied in various systems. In Salmonella, expression of two type III secretion systems involved in adaptation and pathogenesis inside the host showed sensitivity to genome supercoiling (Galán & Curtiss, 1990; O Cróinín et al., 2006). In E. coli, switching of the fimbriae gene was TopoI-dependent (Dove & Dorman, 1994). Similarly, the mutation in topI led to reduced expression of recA in E. coli, conferring increased UV sensitivity (Urios et al., 1990). Moreover, deletion of topI reduced the expression of the fooB regulator required for the expression of fimbriae F165(1) needed for the virulence and pathogenesis of E. coli 4787 (O115 : KV165) (Tessier et al., 2007). Also, a mutation in topI suppressed mutation of the leu-500 promoter (a supercoiling-sensitive promoter), leading to enhanced expression by perturbing the topology of regulatory DNA elements (Pruss & Drlica, 1985). These studies suggest that alteration in topoisomerase activities may lead to changes in global gene expression, with various phenotypic consequences. A detailed characterization of a DNA gyrase mutant in Salmonella suggested a change in global genome supercoiling and resistance to various drugs (Webber et al., 2013). Transcriptome analysis of the mutant revealed altered expression of the genes involved in export, stress and metabolic pathways (Webber et al., 2013), conferring numerous phenotypic changes. In Streptococcus pneumoniae, inhibition of DNA gyrase resulted in the activation of stress-responsive, virulent genes and simultaneous down regulation of several housekeeping genes involved in cell metabolism and growth (Ferrándiz et al., 2010). Thus, previous studies and the present study show that perturbation in topoisomerase activity confers global changes in bacteria leading to an altered phenotype and drug sensitivity.

The diffused nucleoid architecture of the Msm mutant indicated a perturbation in genome supercoiling. The level of supercoiling of the genome may influence initial binding of RNAP holoenzyme and subsequent activation of specific sets of promoters leading to global changes in gene expression. A study in E. coli suggested that the binding of stationary phase-specific σ^38 to the target gene was favoured on a template with low superhelical density, while the binding of housekeeping σ^70 was optimal with the negative supercoiled DNA (Kusano et al., 1996). The altered supercoiling of the bacterial genome may potentially rewire the distribution of different species of holoenzymes on the TUs, leading to a change in global gene expression profile. Moreover, reduction of TopoI activity in bacteria would lead to the accumulation of excessive negative supercoiling that may impede the movement of RNAP and its subsequent distribution on the TUs. The occupancy profile of RNAP on the genes affected in expression in the Msm mutant revealed varied distribution of the RNAP on the TUs. The accumulation of RNAP on the promoter region of the TUs could be a result of excessive negative supercoiling (torsional strain), which in turn may retard the entry of RNAP into the elongation phase. These alterations could impact the global transcription profile of the cell, leading to the changes illustrated. A similar observation was reported in a yeast Topo II mutant in which the distribution of RNAP on TUs was affected upon depletion of Topo II activity (Joshi et al., 2012). A mutation in the gene or reduction in topoisomerase activity in neuronal cells resulted in the accumulation of RNAP on the promoter region of TUs (King et al., 2013).

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**Fig. 8.** Perturbation in TopoI level leads to the changes in various phenotypic characteristics in Msm.
Although different from \textit{Mtb} in various respects, \textit{Msm} is often used as a surrogate host for a variety of studies (Chaturvedi et al., 2007). The use of \textit{Msm} as a surrogate for \textit{Mtb} for drug screening and genetic studies is a matter of debate (Ramón-Garcia et al., 2013). However, in the present study, the ability of Mttopol to functionally complement the \textit{Msm} \textit{topoI} knock-down strain offers an opportunity to carry out genetic studies to better understand the function of Mttopol and for screening the specific inhibitors against the enzyme. Given the faster growth rate of \textit{Msm}, the strain would expedite studies on the \textit{in vivo} function of \textit{Mtb} Topol and exploit it as a drug target.

The importance of Topol, the first described topoisomerase and originally named as omega protein, can be appreciated by the vast body of information on its mutational analysis in \textit{E. coli}. Early genetic studies revealed the requirement of Topol for \textit{E. coli} growth. Deletion of Topol was found to be lethal in the absence of any compensatory mutations in DNA gyrase (DiNardo et al., 1982). Direct evidence of the essential requirement for Topol came from a study using a temperature-sensitive Topol mutant in which inactivation of Topol at 42 °C led to the arrest of cell growth (Zumstein & Wang, 1986). Thus, despite having additional DNA relaxation enzymes (Topo III and Topo IV), Topol function is of critical importance in \textit{E. coli}. In contrast, the Topol null mutants of \textit{Salmonella Typhimurium} and \textit{Shigella flexineri} were found to be viable even in the absence of any compensatory mutations (Ni Bhrain & Dorman, 1993; Trucksis et al., 1981). Different members of the Gammaproteobacteria thus seem to vary in growth and survival when their respective Topol is depleted. In contrast, in both mycobacterial species studied here, Topol appears to be indispensable for cell survival.

To conclude, Topol is essential in different species of mycobacteria irrespective of whether they possess a sole enzyme or have additional DNA relaxation enzymes. Topol-depleted \textit{Msm} strains may offer a unique opportunity and advantage over their \textit{Mtb} counterparts. The importance of Topol seems to vary across eubacterial species, ranging from indispensability to having a specific dedicated role. Nevertheless, both present and previous studies emphasize its critical importance in maintenance of topological homeostasis. Perturbation in Topol expression conferred various phenotypic effects as a consequence of altered distribution of the transcription machinery on TUs. Analysis of the global transcriptome and RNAP distribution on the TUs of the Topol mutant of mycobacteria may facilitate further understanding of the connection between genome supercoiling, gene expression and phenotypic characteristics in this group of organisms.

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