6-29-2017

Protein-protein Interactions of Bacterial Topoisomerase I

Srikanth Banda
sband003@fiu.edu

DOI: 10.25148/etd.FIDC001958

FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

PROTEIN-PROTEIN INTERACTIONS OF BACTERIAL TOPOISOMERASE I

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

by

Srikanth Banda

2017

To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Srikanth Banda, and entitled Protein-protein Interactions of Bacterial Topoisomerase I, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

_____
Yesim Darici

_____
Yuan Liu

_____
Xiaotang Wang

_____
Watson Lees

_____
Yuk-Ching Tse-Dinh, Major Professor

Date of Defense: June 29, 2017

The dissertation of Srikanth Banda is approved.

_____
Dean Michael R. Heithaus
College of Arts, Sciences and Education

_____
Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2017

DEDICATION

I dedicate this dissertation to my mother, and my wife, for their unconditional love and support.

ACKNOWLEDGMENTS

I'm grateful to all the people at FIU, for without their support and encouragement, I would not be able to accomplish much of my work. I'm forever indebted to my advisor, Dr. Yuk-Ching Tse-Dinh, for her constant support and advice. I have learnt a lot from her mentorship, and I'm grateful to have worked under her guidance.

I would like to thank my committee members, Dr. Watson Lees, Dr. Xiaotang Wang, Dr. Yesim Darici and Dr. Yuan Liu, for their guidance and support throughout my Ph.D. training. I would also like to thank Dr. Prem Chapagain, Dr. Jin He, Dr. Xuewen Wang for their guidance. I would like to thank Dr. Purushottam Babu Tiwari for his help with the SPR experiments.

I would like to thank all my peers, and lab members for their support and advise in my research. I would like to thank Dr. Arasu Annamalai for patiently advising and guiding me in the lab. I would like to thank Qingxuan Zhou, Nan Cao, Pamela Gracia Moreno, Shayna Sandhaus, Wenjie Wang, Jose Chang, Angelo Andres, as well as other past and present lab members for their help and support.

I'm grateful for the Research assistantship provided by FIU College of Arts, Sciences and Education through the School of Integrated Science and Humanity (SISH).

ABSTRACT OF THE DISSERTATION

PROTEIN-PROTEIN INTERACTIONS OF BACTERIAL TOPOISOMERASE I

by

Srikanth Banda

Florida International University, 2017

Miami, Florida

Professor Yuk-Ching Tse-Dinh, Major Professor

Protein-protein interactions (PPIs) are essential features of cellular processes including DNA replication, transcription, translation, recombination, and repair. In my study, the protein interactions of bacterial DNA topoisomerase I, an essential enzyme, were investigated. The topoisomerase I in bacteria relaxes excess negative supercoiling on DNA, and maintains genomic stability. Investigating the PPI network of DNA topoisomerase I can further our understanding of the various functional roles of this enzyme. My study is focused on topoisomerase I of *Escherichia coli* and *Mycobacterium smegmatis*. Firstly, we have explored the biochemical mechanisms for an interaction between RNA Polymerase, and topoisomerase I in *E. coli*. Molecular docking, and molecular dynamic simulations have predicted that the interactions are mediated through electrostatic, and hydrogen bonding. The predicted Lysine residues (K627, K664) of topoisomerase I that are involved in the electrostatic interactions were mutated to Alanine, and its effect on the binding efficiency with RNA polymerase was reported. In a separate study, PPI partners of topoisomerase I in mycobacteria were identified. Knowledge gained from the study can provide valuable insights into the physiological functions of a validated drug target, DNA topoisomerase I, in pathogenic mycobacteria. Co-immunoprecipitation, and pull-down

assays were coupled to mass spectrometry for identification of the protein partners of mycobacterial topoisomerase I. The study has identified RNA polymerase, and putative helicases (DEAD/DEAH BOX helicases) as potential protein partners of mycobacterial topoisomerase I. My results indicated that the tail region of the CTD-topoisomerase I was required for direct physical interaction with the RNAP beta' subunit. My studies have also verified the physiological relevance of the topoisomerase I - RNA polymerase interactions for survival under antibiotic, and oxidative stress. Lastly, I report a direct physical interaction between *E. coli* topoisomerase I and RecA by pull-down assays. Previous studies have shown that RecA, a DNA repair protein, can stimulate the relaxation activity of *E. coli* topoisomerase I. Our new results showed that the stimulatory effect can be attributed to the physical interaction of topoisomerase I with RecA.

TABLE OF CONTENTS

CHAPTER PAGE

INTRODUCTION 1
A. TOPOISOMERASES AND GENOME STABILITY 1
B. TOPOISOMERASES AS THERAPEUTIC TARGETS……9
C. PROTEIN-PROTEIN INTERACTIONS; CROSS-TALK BETWEEN PROTEINS...19
D. BACTERIAL TOPOISOMERASE I……26

OVERVIEW 30

CHAPTER 1: MOLECULAR MECHANISMS OF INTERACTION BETWEEN *E. COLI* RNA POLYMERASE AND TOPOISOMERASE I 32
ABSTRACT.....32
INTRODUCTION 33
MATERIALS AND METHODS.....35
RESULTS AND DISCUSSION.....41
CONCLUSIONS 44

CHAPTER 2: IDENTIFICATION OF PROTEIN PARTNERS OF MYCOBACTERIAL TOPOISOMERASE I.....49
ABSTRACT.....49
INTRODUCTION 50
MATERIALS AND METHODS.....54
RESULTS……..60
DISCUSSION.....71

CHAPTER 3: EVOLUTION OF A NOVEL MECHANISM FOR MYCOBACTERIAL RNA POLYMERASE AND TOPOISOMERASE I INTERACTION 74
ABSTRACT.....74
INTRODUCTION 75
MATERIALS AND METHODS.....76
RESULTS 90
DISCUSSION.....104

CHAPTER 4: INVESTIGATING A DIRECT INTERACTION BETWEEN *ESCHERICHIA COLI* TOPOISOMERASE I AND RECA 107
ABSTRACT.....107
INTRODUCTION 107
MATERIALS AND METHODS.....111
RESULTS 117
DISCUSSION……..126

SUMMARY AND FUTURE WORK 128

REFERENCES……………………………..130

VITA 153

LIST OF TABLES

TABLE PAGE

INTRODUCTION

I. T1 Classification of topoisomerases…….8

I. T2 Inhibitors of topoisomerases…….18

CHAPTER 1

1. T1. Primers used in Site-Directed Mutagenesis…….36

CHAPTER 2

2. T1. PCR primers for Gibson cloning…….56

2. T2. Potential protein partners of topoisomerase I identified by Co-IP/MS…...65

2. T3. Potential protein partners of topoisomerase I identified by Pull-down/MS……66

CHAPTER 3

3. T1. Strains used in this study…….78

3. T2. PCR primers for Gibson cloning…….79

3. T3. Primers used for Site-Directed Mutagenesis……..…….80

3. T4. Recombinant proteins used in the study…….85

CHAPTER 4

4. T1. Potential amino acid residues for the formation of hydrogen bonds during EcTopoI-RecA complex formation……..123

4. T2. Potential amino acid residues for the formation of salt bridges during EcTopoI-RecA complex formation……..123

LIST OF FIGURES

FIGURE PAGE

INTRODUCTION

I.1. Domain organization of type IA topoisomerases……..3

I.2. Type I topoisomerases as modulators of DNA topology…….……2

I.3. Domain structures of type II topoisomerases……....6

I.4. Type II topoisomerases as modulators of DNA topology…….……7

I.5. Coordination of topoisomerases in the DNA replication, and transcription processes…….....9

I.6. Covalent cleavage complex via transesterification reaction……10

I.7. Repair pathways for the removal of topoisomerase-DNA complexes……11

I.8. Formation of a Drug-DNA-Topoisomerase II ternary complex……..12

I.9. Crystal structure of human topoisomerase II β (core) in complex with DNA, and Etopoisode……13

I.10. Crystal structure of M. tuberculosis GyrBA core in complex with DNA and moxifloxacin……...15

I.11. Crystal structure of Acinetobacter baumannii topoisomerase IV in complex with moxifloxacin and DNA……...16

I.12. Different types of PPIs based on their binding affinities……...20

I.13. Results from a STRING search of topoisomerase I (M. tuberculosis H37v)……..21

I.14. Schematic structures of known toposome with their in vivo roles……24

I.15. Proposed enzyme bridged strand passage mechanism of relaxation by bacterial topoisomerase I……...26

I.16. Crystal structures of bacterial topoisomerase I……..28

CHAPTER 1

1.1. MD simulation of mutant EcTopoI (K664A) in complex with RNAP at different times of the simulation trajectory……43

1.2. Association of RNAP-EcTopoI……..46

1.3. Residues involved in the EcTopoI-RNAP complex formation……...47

1.4. Lysine 627,664 are essential for the complex formation with RNAP……48

CHAPTER 2

2.1. A comparison of type IA topoisomerases showing highly-conserved N-terminal domains (NTD), and variable C-terminal domains (CTD)…….....53

2.2. Co-immunoprecipitation of topoisomerase I and interacting proteins from M. smegmatis lysate……...63

2.3. Pull-down assay of MsmTopoI-RNAP interaction with HisPur Cobalt agarose resin……...64

2.4. Unique peptides of RNAP subunits (highlighted) identified by mass spectrometry of the eluates from the Co-IP assay…….67

2.5. Physical interactions of putative DEAD/DEAH Box helicase with topoisomerase I……..68

2.6. Putative helicase, RhlE, has a 3'-5' DNA helicase activity……69

2.7. Putative helicase-RhlE and M. tuberculosis topoisomerase I (MtbTopoI) have a mutual inhibitory effect on their activities…….70

CHAPTER 3

3.1. Protein (M. smegmatis topoisomerase I) purification…….83

3.2. Relaxation activity of the purified topoisomerase I……84

3.3. Cross-reactivity of Rabbit MtbTopoI polyclonal antibodies with MsmTopoI……..86

3.4. Reverse pull-down of MsmTopoI from M. smegmatis soluble lysate with purified recombinant RNA polymerase β' subunit (N-terminal His-tagged).…...91

3.5. M. tuberculosis H37Rv topoisomerase I and RNA polymerase are protein-protein interaction partners……92

3.6. (A) Direct physical interaction between purified M. smegmatis topoisomerase I and RNA polymerase β'subunit……..96

3.6. (B, C) Direct physical interaction between purified *M. smegmatis* topoisomerase I and RNA polymerase β'subunit……..97

3.7. Identification of MsmTopoI domain required for interaction with RNAP.....98

3.8. Mapping of MsmTopoI sequence required for interaction with RNAP…....99

3.9. Inhibition of MsmTopoI-RNAP interaction by overexpression of recombinant MsmTopoI-CTD……100

3.10. Effect of overexpressing the CTD of MsmTopoI on growth……..101

3.11. Effect of TopoI-CTD overexpression on sensitivity of M. smegmatis to stress challenge……102

3.12. The C-terminal tail of topoisomerase I from actinobacteria is rich in basic amino acids……103

CHAPTER 4

4.1. Multiple activities of E. coli RecA……...110

4.2. Purification of His-tagged E. coli topoisomerase I and its NTD……..113

4.3. Direct physical interaction between purified E. coli RecA and topoisomerase I……118

4.4. ATP promotes binding of E. coli RecA to topoisomerase I…….121

4.5. RecA-EcTopoI complex predicted by molecular docking……122

4.6. NTD of EcTopoI can interact with RecA as efficiently as full-length EcTopoI……124

4.7. Pull-down of RecA from E. coli lysates by EcTopoI and NTD-EcTopoI…126

ABBRIEVIATIONS AND ACRONYMNS

ARF	Human ADP ribosylation factor
ATP	Adenosine triphosphate
ATCC	American Type Culture Collection
ARG	Arginine
CcTopII	Coprinus cinereus DNA topoisomerase II
CTD	C-terminal domain
Co-IP	Co-Immunoprecipitaton assay
E. coli	*Escherichia coli*
EcTopoI	*Escherichia coli* topoisomerase I
EDTA	Ethylenediaminetetraacetic acid
fs	femtosecond
GLU	Glutamic acid
HCl	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
HIS	Histidine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IgG	Immunoglobulin G
γ-^{32}P	Adenosine triphosphate, labeled on the gamma phosphate group with ^{32}P
K_D	Equilibrium dissociation constant
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate

k_a	Association rate constant
k_d	Dissociation rate constant
LB	Luria broth
LYS	Lysine
MsmTopoI	*Mycobacterium smegmatis* DNA topoisomerase I
MtbTopoI	*Mycobacterium tuberculosis* H37Rv DNA topoisomerase I
MD	Molecular dynamic simulations
$MgCl_2$	Magnesium chloride
Mocr	monomer of a bacteriophage T7 protein, Ocr
MDR	Multi Drug Resistant
NTD	N-terminal domain
NaCl	Sodium chloride
NVT	Constant number of particles, Volume, and Temperature
ns	nanosecond
OD_{600}	Optical density or absorbance at wavelength of 600 nm.
PPI(s)	Protein-protein interaction(s)
PDB	Protein data bank
PCR	Polymerase chain reaction
PAGE	Polyacrylamide gel electrophoresis
RNAP	RNA polymerase
RPOB	RNA polymerase β subunit
RPOC	RNA polymerase β’ subunit
ssDNA	single stranded DNA

STRING	search tool for recurring instances of neighboring genes
SPR	Surface plasmon resonance
SDS	Sodium dodecyl sulfate
TopoI	DNA topoisomerase I
TDR	Totally Drug Resistant
TB	Tuberculosis
VMD	Visual molecular dynamics
Zn^{2+} or Zn (II)	Zinc (divalent cation)

INTRODUCTION

A. TOPOISOMERASES AND GENOME STABILITY

The DNA topoisomerases have been termed "the magicians of the DNA world" as they resolve all the topological problems of DNA (Wang, 2002). Ever since the discovery of topoisomerases in *Escherichia coli* by James Wang, considerable research has been conducted to study the implications of these enzymes on the essential cellular processes. James. C. Wang treated covalently closed circular DNA duplexes containing negative supercoils with a purified *E. coli* protein ω and observed a partial loss of the supercoiling (Wang, 1971). He also observed that the protein is intrinsically carrying out the functions of endonucleases (nicking the DNA) and DNA ligases (Resealing the DNA). The protein, ω, is now referred to as topoisomerase I. Later, James Champoux discovered that a mouse nuclear extract could remove the supercoils from closed circular polyoma virus DNA by nicking and sealing activities (Champoux & Dulbecco, 1972), hinting at the possibility of the existence of enzymes in eukaryotic cells identical to those of the prokaryotic topoisomerase I. The common ground for the enzymes/proteins discovered by Wang and Champoux was their ability to relax the supercoiled DNA (Champoux, 2001; Wang, 2002). However, it was postulated that the mechanism of relaxing the supercoiled DNA by the prokaryotic topoisomerase I is different from its eukaryotic counter-part (Maxwell & Gellert, 1986). The prokaryotic enzyme discovered by Wang is now categorized as a type IA topoisomerase, and the eukaryotic enzyme discovered by Champoux is classified under type IB. Martin Gellert and co-workers discovered DNA gyrase, a prokaryotic DNA topoisomerase II that could introduce supercoils, during a search for the co-factors of the

host that support site-specific recombination by bacteriophage λ (Gellert et al., 1976). Studies on archaea, a third domain of life, apart from eukaryotes and prokaryotes, have led to the discovery of reverse gyrase (Kikuchi & Asai, 1984), DNA topoisomerase V (Slesarev, 1993) and DNA topoisomerase VI (Bergerat et al., 1997). Reverse gyrase, a unique enzyme found only in the thermophiles have been reported to introduce positive supercoils to a relaxed DNA molecule (Declais et al., 2001). The currently discovered topoisomerases fall in to two categories: type I and type II (Table I. T1). The type I topoisomerases transiently cleave a single strand of the DNA in an ATP-independent manner, whereas the type II topoisomerases transiently break both strands of the DNA in an ATP-dependent manner.

The type I topoisomerases were further subdivided in to type IA, and IB based on the polarity of strand cleavage. The activities of type I topoisomerases are depicted in figure I. 2. Type IA enzymes form a transient 5'-phosphotyrosine covalent complex and releases a free 3'-OH end whereas the type IB forms a 3'-phosphotyrosine covalent complex and produces a free 5'-OH end. Another differentiation is that the type IA promotes the passage of the intact strand through the broken strand (strand passage), whereas the type IB allows the broken strand to freely rotate around the intact strand. Type IA topoisomerases are composed of two domains: a conserved core domain that is involved in the trans-esterification reactions, and a carboxyl-terminal domain highly variable in size and sequence (Figure I. 1) (Viard et al., 2007). Type IA topoisomerases are present in bacteria (topoisomerase I, III), archaea (reverse gyrase) and eukarya (topoisomerase IIIα and topoisomerase IIIβ). Type IB topoisomerases, DNA swivelases, fundamentally differ in the structure and mechanism from type IA topoisomerases (Redinbo et al., 1998). Type IB

topoisomerases are ubiquitous in eukaryotes, with some homologues scattered among a few viruses and bacteria (Krogh & Shuman, 2002). The type IB enzymes have a preferential binding for positively or negatively supercoiled DNA substrates rather than the relaxed substrates (Madden et al., 1995; Frohlich et al., 2007).

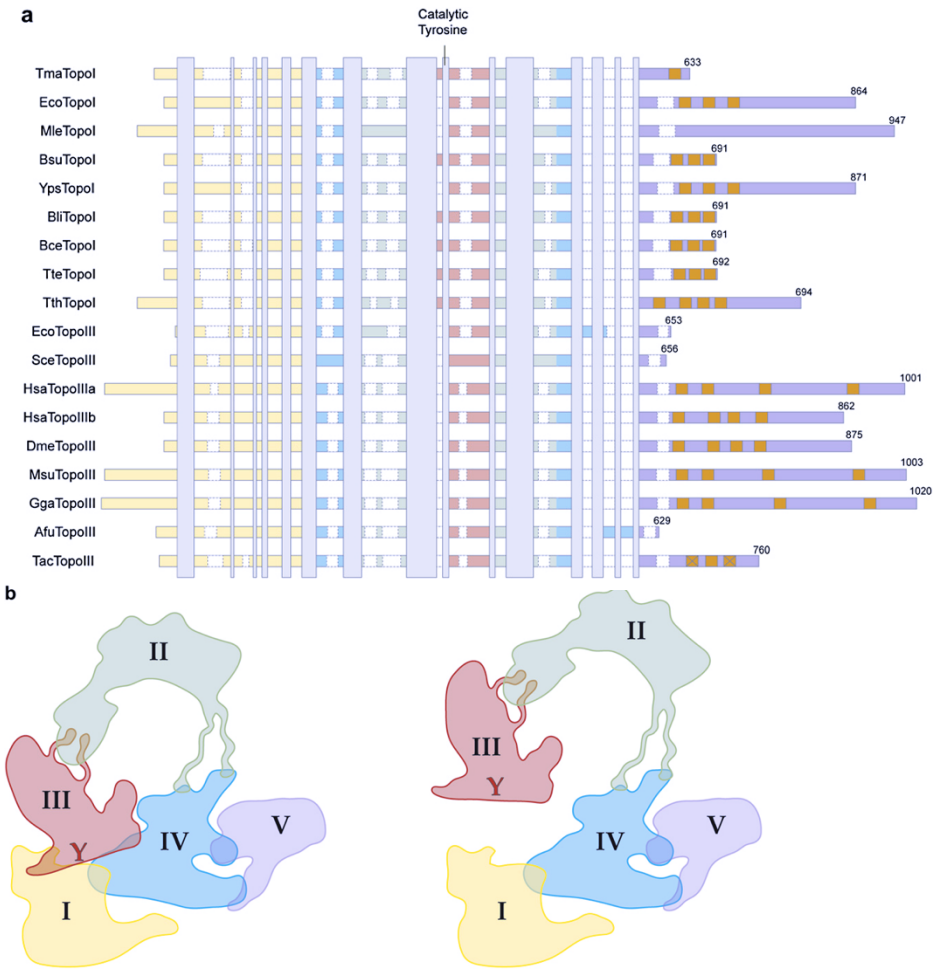


Figure I. 1. Domain organization of type IA topoisomerases (Viard et al., 2007). (a)Sequence alignment of type IA topoisomerases across different organisms. The conserved sequences are represented by foreground gray boxes. Orange boxes: Putative tetracysteine zinc finger motifs. Crossed orange boxes: Putative tetracysteine zinc finger motifs with one or two histidines instead of cysteines. The domains are color coded: domain I (yellow), domain II (green), domain III (red), domain IV (blue), domain V (purple). Refer to the abbreviations section for the description of enzyme abbreviations. (b) A general schematic of an open and closed structure of type IA topoisomerases. The active site tyrosine (Y, red colored) is in domain III.

The structural and sequential differences between type IA and type IB have confirmed the independent origins of these enzymes (Forterre et al., 2007). Most of the type I topoisomerases exist as monomeric units, formed by a single topoisomerase domain. However, reverse gyrase and Topoisomerase V are exceptions, as they have a topoisomerase domain bound to a helicase-like domain in the case of reverse gyrase and DNA repair-like domain in the case of Topoisomerase V. Classified under type IC family, Topoisomerase V has a mechanism of action that is similar to that of type IB enzymes. However, topoisomerase V is distinct in having no sequence or structural similarities with other topoisomerases (Baker et al., 2009). Strikingly, topoisomerase V is a bifunctional enzyme, carrying out the functions of DNA relaxation through its N-terminal (Slesarev et al., 1993) and DNA repair via its C-terminal (Belova et al., 2001).

E. coli topoisomerase I, a type IA topoisomerase, can only relax negative supercoils. Whereas the eukaryotic topoisomerase I, a type IB topoisomerase, can relax both negative and positive supercoils (Champoux & Dulbecco, 1972; Pommier 2010). The DNA topoisomerase I or topoisomerase III deletion mutants of *E. coli* are viable, suggesting an apparent non-essentiality of these enzymes in *E. coli* (Stupina & Wang., 2005). However, each organism must have at least one type IA topoisomerase activity to be viable. The potential for bacterial topoisomerase I to form a stable complex with DNA, leading to the SOS response and eventual cell death has made this enzyme a potential antibacterial drug target (Cheng et al., 2005). Unlike in *E. coli*, the topoisomerase I of *M. tuberculosis* and *M. smegmatis* was reported to be indispensable because there is no other type IA topoisomerase present in mycobacteria (Ahmed et al., 2014, 2015). Currently, there are no commercially available antibacterial agents targeting bacterial topoisomerase I.

Efforts to identify and develop novel drugs against bacterial topoisomerase I are on the rise. Human topoisomerase I, on the other hand, is a well-established target for cancer chemotherapy. Camptothecin and its analogs are being widely used clinically to target the human topoisomerase I (Wall & Wani., 1995).

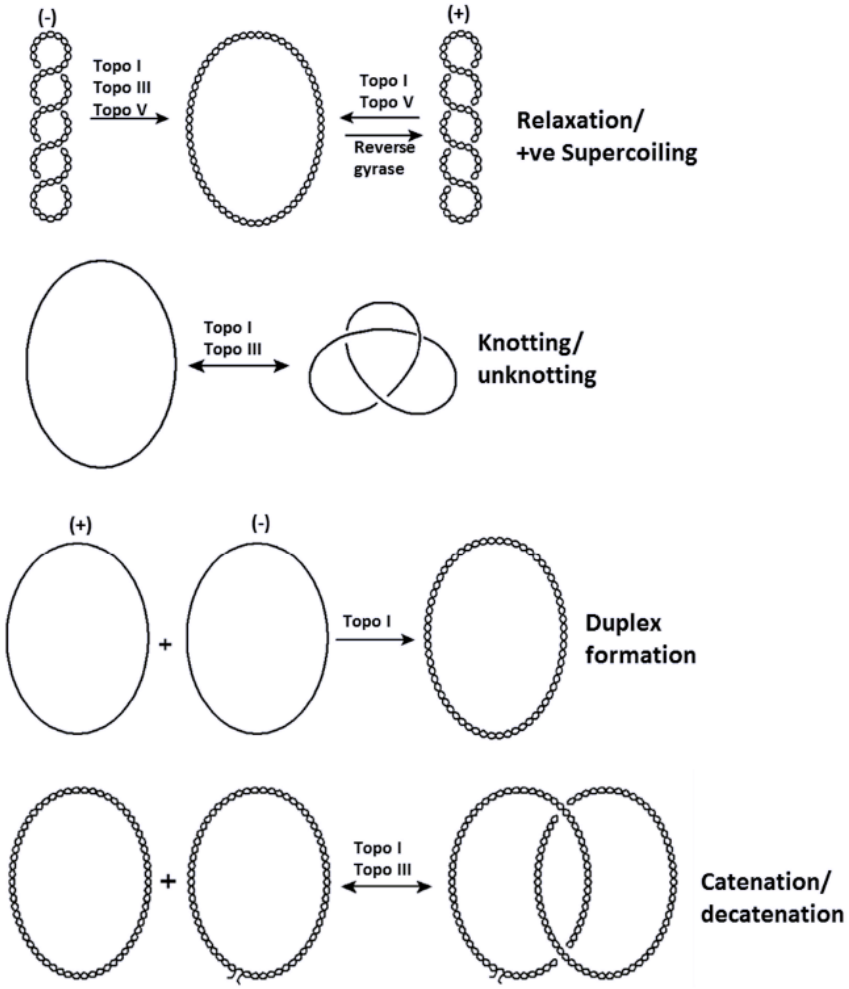


Figure I. 2. Type I topoisomerases as modulators of DNA Topology (Bush et al., 2015). Type I topoisomerases can catalyze the relaxation of a supercoiled DNA. Reverse gyrase can introduce positive supercoils in the DNA. Knotting/unknotting, catenation/decatenation, and duplex formation of DNA are also catalyzed by the type I topoisomerases.

Type II topoisomerases have been reported in all proliferating cells and are essential for the survival of all species (Watt & Hickson, 1994; Wang, 1996). Type II topoisomerases in bacteria are represented by DNA gyrase and topoisomerase IV. In eukaryotes, type II topoisomerases are represented by two closely related isoforms, topoisomerase IIα and topoisomerase IIβ. The isoforms are encoded by different genes, although displaying a high degree (70%) of amino acid sequence identity (Champoux, 2001; McClendon & Osheroff, 2007). Topoisomerase IIα is indispensable as it is essential for the survival of actively growing cells. Topoisomerase IIβ, although dispensable the cellular level, is required for the neural development in mice (Yang et al., 2000). Type II topoisomerases, in contrast to type I topoisomerases, are multimeric enzymes (Figure I. 3). Each monomer of the enzyme has an ATP binding domain, and an active site responsible for the cleavage-religation of a single strand of DNA.

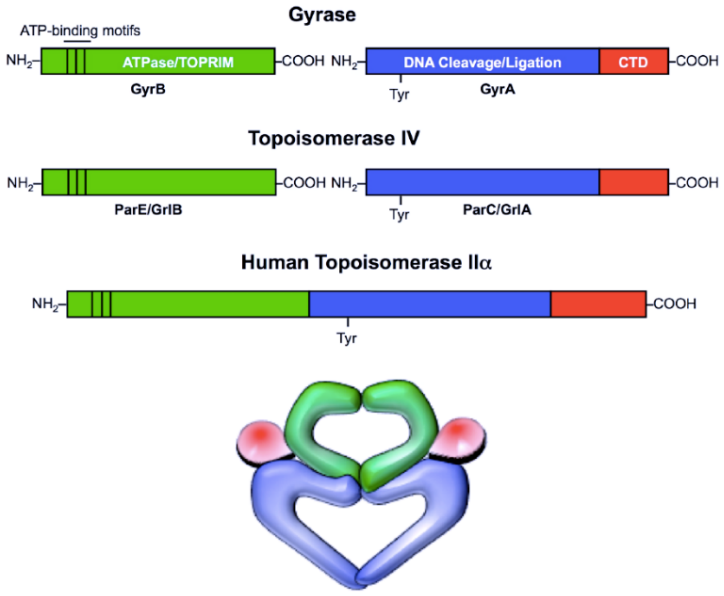


Figure I. 3. Domain structures of type II topoisomerases (Aldred et al., 2014)

Chromosomal segregation after DNA replication, and before cell division requires topoisomerase II activity (Hartsuiker et al., 1998). Therefore, topoisomerase II is indispensable for cell survival, and can serve as a potential drug target in cancer and antibiotic therapies. The activities of type II topoisomerases are depicted in figure I. 4. The cellular levels of topoisomerases have a direct relation with the growth rates, and mitotic failure is reported when the cellular levels drop below the threshold (Nitiss, 1998).

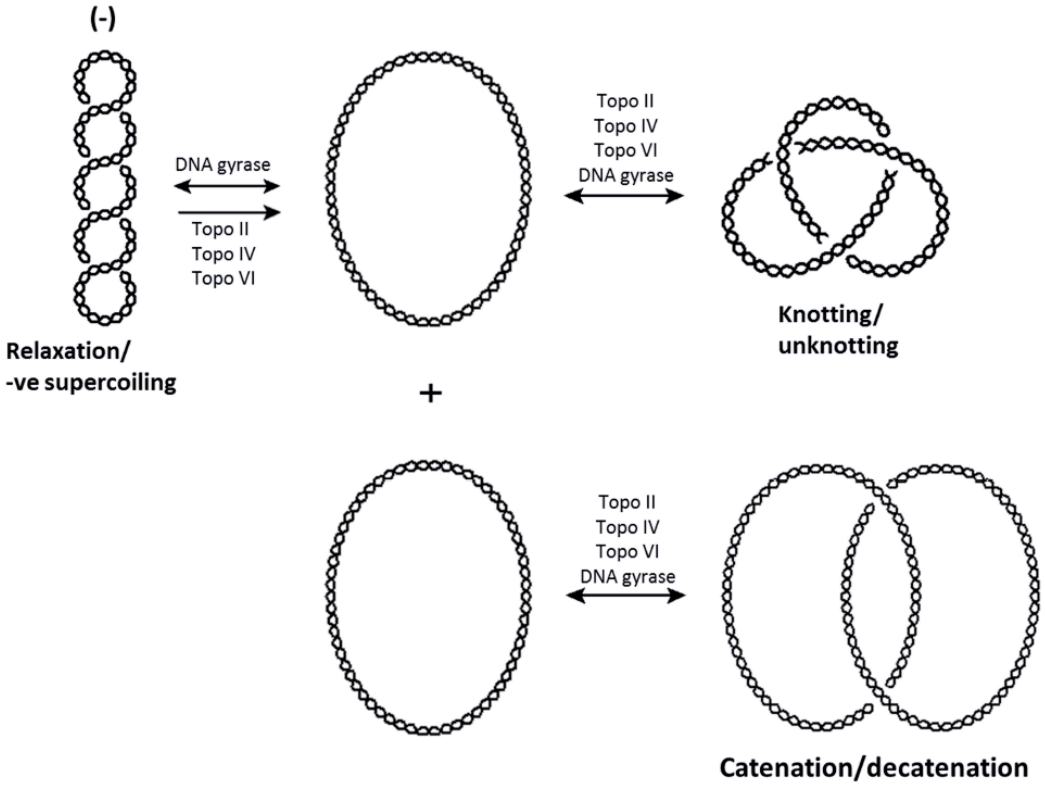


Figure I. 4. Type II topoisomerases as modulators of DNA Topology (Bush et al., 2015). Type II topoisomerases can catalyze the relaxation of a supercoiled DNA, apart from catenation/decatenation, and knotting/unknotting of DNA. Gyrase can also introduce supercoils into DNA. Here, only the relaxation of negatively supercoiled DNA by type II topoisomerases is shown. However, it should be noted that all the known type II topoisomerases can also relax positively supercoiled DNA.

Table I. T1. Classification of Topoisomerases

Subfamily	Representative members	Mechanism of Action	ATP dependence
IA	Bacterial DNA topoisomerase I, III Yeast DNA topoisomerase III *Drosophila melanogaster* DNA topoisomerases IIIα, IIIβ Mammalian DNA topoisomerases IIIα, IIIβ Reverse Gyrase (Thermophilic Archaea)	Strand passage	No (Reverse Gyrase is an exception)
IB	Eukaryotic DNA topoisomerase I Mammalian mitochondrial DNA topoisomerase I Pox virus topoisomerase	Swivel	No
IC	*Methanopyrus kandleri* DNA topoisomerase V	Swivel	No
IIA	Bacterial gyrase, DNA topoisomerase IV Phage T4 DNA topoisomerase. Yeast DNA topoisomerase II Drosophila DNA topoisomerase II Mammalian DNA topoisomerases IIα, IIβ	Strand passage	Yes
IIB	*Sulfolobus shibatae* DNA topoisomerase VI	Strand passage	Yes

B. TOPOISOMERASES AS THERAPEUTIC TARGETS

Topoisomerases have garnered attention of the scientific community for their potential as chemotherapeutic and antibiotic drug targets. Topoisomerases are the caretakers of the genome. Multitudes of topoisomerases coordinate the essential processes such as replication, transcription, recombination, and repair (Figure I.5). Topoisomerases address the supercoiling problems by cleaving (single or double strand), and religating the cleaved DNA. During the cleavage process, the enzyme and DNA are linked as covalent complexes momentarily. The covalent complex serves as a potential drug target (Pommier et al., 2010).

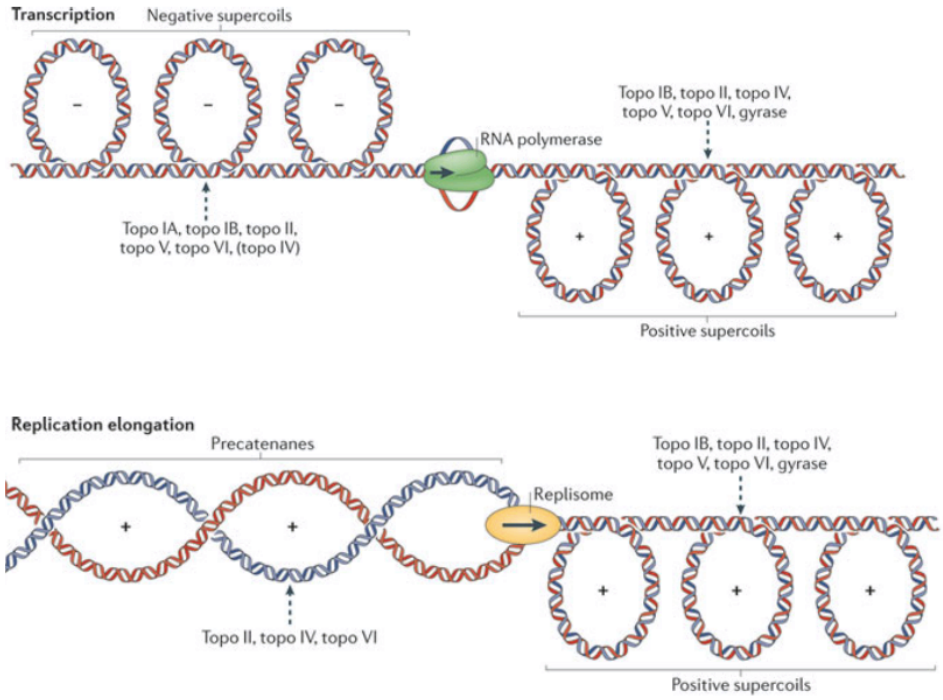


Figure I. 5. Coordination of topoisomerases in the DNA replication, and transcription processes (Vos et al., 2011)

The tyrosine of the enzyme's active site attacks the phosphorous of DNA, forming a phosphotyrosine covalent complex and thereby, breaking the phospho-diester back bone

of a single strand of DNA. A strand passage (type IA, IIA, IIB) or swivel mechanism (type IB), depending on the enzyme's subfamily (Table I. T1), will precede the religation step (Figure I.6). A second trans-esterification reaction allows the religation of the DNA strand. However, in the case of second trans-esterification, the oxygen of the DNA hydroxyl group (generated in the cleavage reaction) attacks the phosphorous of the phosphotyrosine link, and breaks the covalent bond between the enzyme and DNA (Wang, 2002).

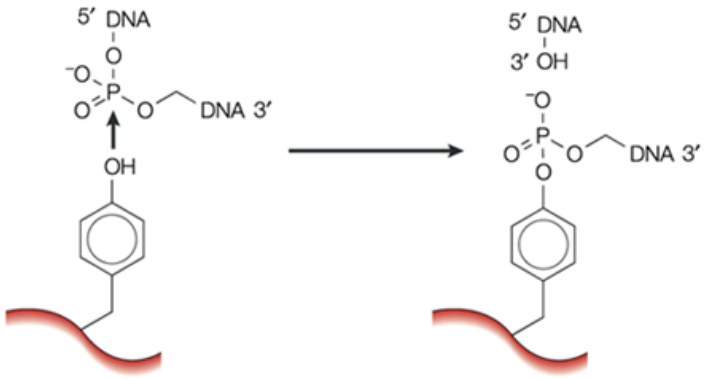


Figure I.6. Covalent cleavage complex via transesterification reaction (Wang, 2002)**.** The reaction represented here is relevant for the transesterification reactions mediated by the type IA, and type II topoisomerases. A reversal of the reaction causes the religation of the cleaved DNA strand. Type IB topoisomerase reactions (not shown here) are mediated via complex formation of the active site tyrosine with the 3'-phosphoryl group.

Insights into the DNA relaxation mechanisms of topoisomerases have paved the way for design and identification of drugs that target either the covalent DNA-enzyme complexes or the catalytic activity of the enzyme (Table I.T2). The drugs have been categorized into two different classes, "topoisomerase poison inhibitors or catalytic inhibitors", depending on their mode of action. Topoisomerase poison inhibitors essentially convert the enzymes into cellular toxins by increasing the concentrations of enzyme-linked

DNA breaks in treated cells and hence, the name topoisomerase poisons (Pommier, 2013). The catalytic inhibitors of topoisomerases, on the other hand, block the overall activity of the topoisomerase, causing the enzyme to lose its functionality (Fortune et al., 2000). Catalytic inhibitors are only effective if the cellular activity of the enzymes is nearly completely inhibited, whereas poison inhibitors can trigger cell death by trapping a small subset of topoisomerase mediated covalent complexes (Aedo & Tse-Dinh, 2012).

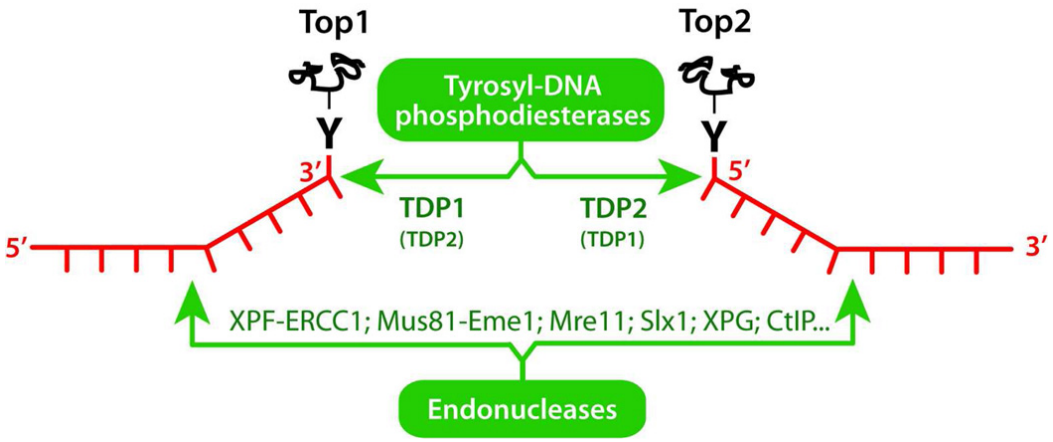


Figure I. 7. Repair pathways for the removal of topoisomerase-DNA complexes (Pommier, 2013)

Eukaryotic topoisomerase I or topoisomerase II serve as targets for drugs used in cancer therapy. For instance, Camptothecin, a plant alkaloid with a potential for anti-cancer activity has topoisomerase I as its only known target. Camptothecin and its derivatives (Irinotecan, Topotecan), reportedly trap the covalent complex of DNA and topoisomerase I, thereby inhibiting the religation step (Pommier et al., 2005). As the cleavage complexes are trapped by Camptothecin, DNA repair pathways (Figure I.7) may intervene. Hence, a combination therapy would be a preferred alternative to using Camptothecin alone. Camptothecin in combination with drugs that target the topoisomerase I induced DNA

damage repair pathways are gaining popularity over the use of the Camptothecin derivatives alone (Zhang et al., 2011; Pommier, 2013). Like topoisomerase I-mediated cell killing, drugs targeting the topoisomerase II activities have also been discovered. Topoisomerase II, essentially, creates a double strand break in the cellular DNA and these breaks as such do not pose a threat to the genomic stability under normal physiological conditions, as the breaks are short lived (Wang, 1996). However, if the covalent complexes of topoisomerase II and DNA are trapped, the concentrations of covalent complexes rise significantly, and apoptotic pathways are triggered. Genomic aberrations can also arise from the insertions, deletions and illegitimate recombination as a result of the topoisomerase-mediated DNA breaks (Fortune et al., 2000).

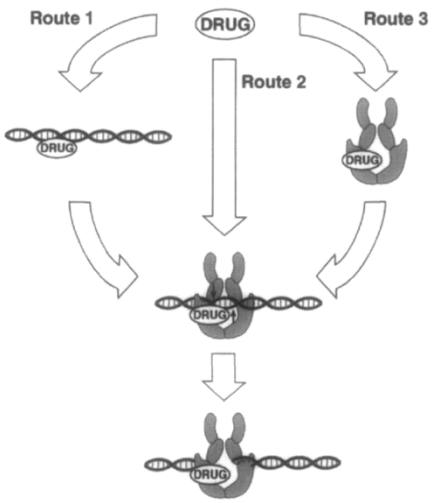


Figure I. 8. Formation of a Drug-DNA-Topoisomerase II ternary complex
(Fortune et al., 2000)

Eukaryotic topoisomerase II is a potential target for the most successful anticancer drugs used clinically in the treatment of human malignancies (Pommier, 1997). The drugs are structurally diverse, but share a common feature in their aromatic core interacting with

DNA in the enzyme-drug-DNA ternary complex (Figure I.8) (MacDonald et al., 1991). Identification of the anticancer drug interaction domains of topoisomerase II from three-dimensional structure of the drug-stabilized ternary cleavage complex can provide a breakthrough in development of chemotherapeutic drugs. A crystal structure of the human topoisomerase II β isoform (core) in complex with the DNA, and an anticancer drug etoposide has been reported (Figure I.9) (Wu et al., 2011). The crystal structure information has provided insights into the binding modes of etoposide, and has also opened avenues for future drug development.

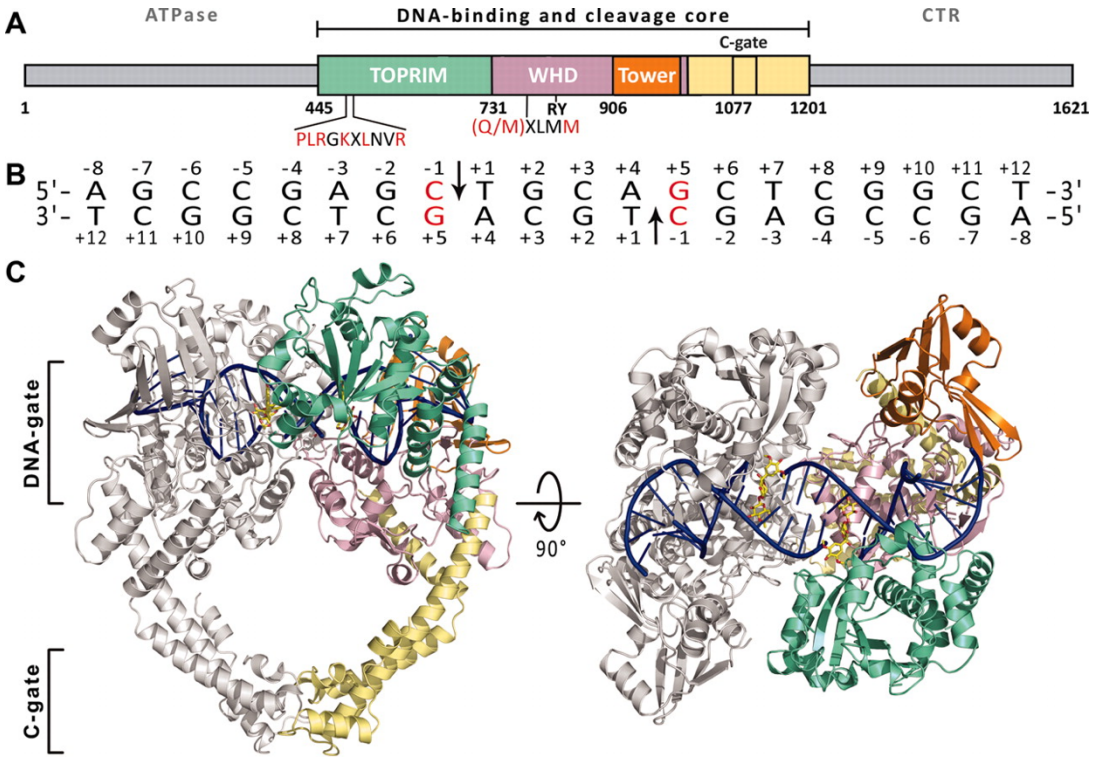


Figure I.9. Crystal structure of human topoisomerase II β (core) in complex with DNA, and Etoposide (Wu et al., 2011). (A) Linear domain organization of human topoisomerase II β. The DNA binding and cleavage core was used in the study. (B) The palindromic DNA substrate used for crystallization studies. The arrows are indicative of the cleavage sites. (C) Orthogonal views of the crystal.

Prokaryotic topoisomerase II enzymes, DNA gyrase and topoisomerase IV, are targets for two classes of antibiotic drugs: quinolones and coumarins. These drugs are effective against pathogens causing tuberculosis, pneumonia and malaria. Fluoroquinolones (Moxifloxacin, Gatifloxacin, Oflaxacin, Levofloxacin etc.,) are proven to be the most successful agents against bacterial pathogens including tuberculosis (TB). Development of fluoroquinolone resistance has been a concern, especially when it is administered as monotherapy (Ginsberg et al., 2003). There is an urgent need for the development of drugs treating the multidrug-resistant and extensively drug-resistant tuberculosis (Gunther, 2014).

A three-drug regimen, moxifloxacin in combination with pretomanid and pyrazinamide, was reported to be effective against TB and MDR-TB compared to the currently used regimen (Dawson & Diacon, 2013). Fluoroquinolones are involved in forming complexes with gyrase and DNA, resulting in the poisoning of *M. tuberculosis*. The development of crystal structures of quinolones in complex with gyrase-DNA, and topoisomerase IV-DNA has advanced our understanding of the quinolone resistance mutations. Structural information of these complexes can provide valuable insights for the development of new quinolone agents. The crystal structure has revealed the importance of water shell of an associated magnesium ion in forming a bridge between fluoroquinolone and gyrase (Figure I.10) (Blower et al., 2016). Quinolones also have a secondary target, topoisomerase IV, in some bacteria. In *Staphylococcus aureus*, quinolones primarily target topoisomerase IV. The crystal structure of *Acinetobacter baumannii* topoisomerase IV complexed with DNA and moxifloxacin shows the wedge-shaped quinolone stacking between the DNA base pairs at the cleavage site. The quinolone stacked in the DNA can

bind the conserved residues of topoisomerase IV DNA cleavage domain via chelation of noncatalytic magnesium ion (Figure I.11) (Wohlkonig et al., 2010). The aromatic rings of quinolone are involved in DNA base stacking at the cleavage site, and at the same time the quinolone is interacting with the residues of the both subunits of the enzyme for creating a cleavage complex, which has been a common theme among the complexes crystalized thus far.

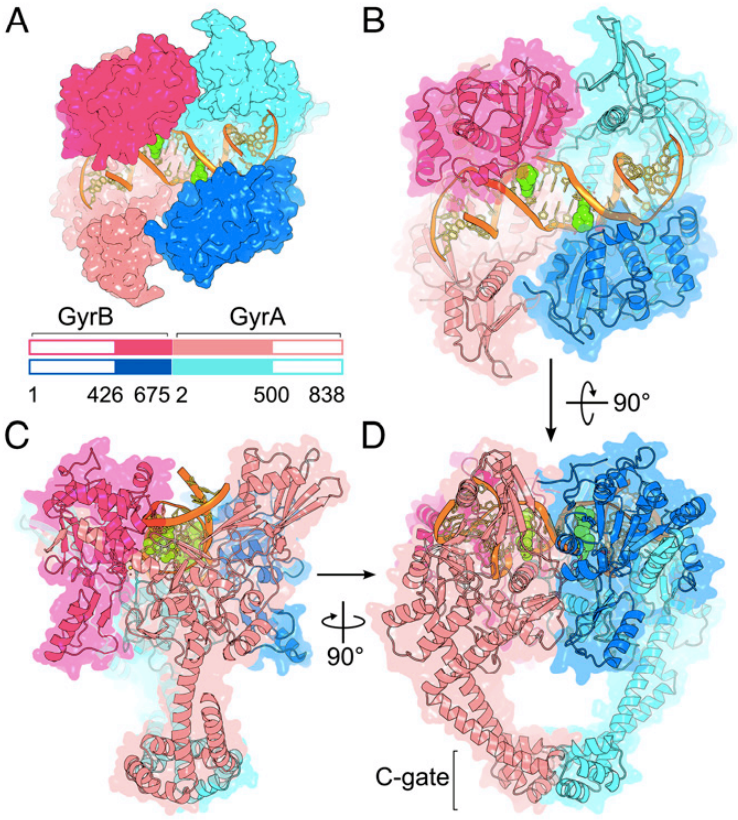


Figure I.10. Crystal structure of *M. tuberculosis* GyrBA core in complex with DNA and moxifloxacin (Blower et al., 2016). (A) The GyrBA fusion comprises of amino acids 426-675 of GyrB, and amino acids 2-500 of GyrA. The fused protein is shown in two shades of pink and blue. The DNA is represented in orange. Magnesium ions are shown as yellow spheres, and Moxifloxacin is shown by green spheres. (B-D) Orthogonal views of the complex.

The overuse of quinolones has resulted in a rise in the quinolone-resistant bacterial strains. Target-mediated resistance, plasmid-mediated resistance, or chromosome-mediated resistance are the possible mechanisms for development of quinolone resistance (Aldred et al., 2014).

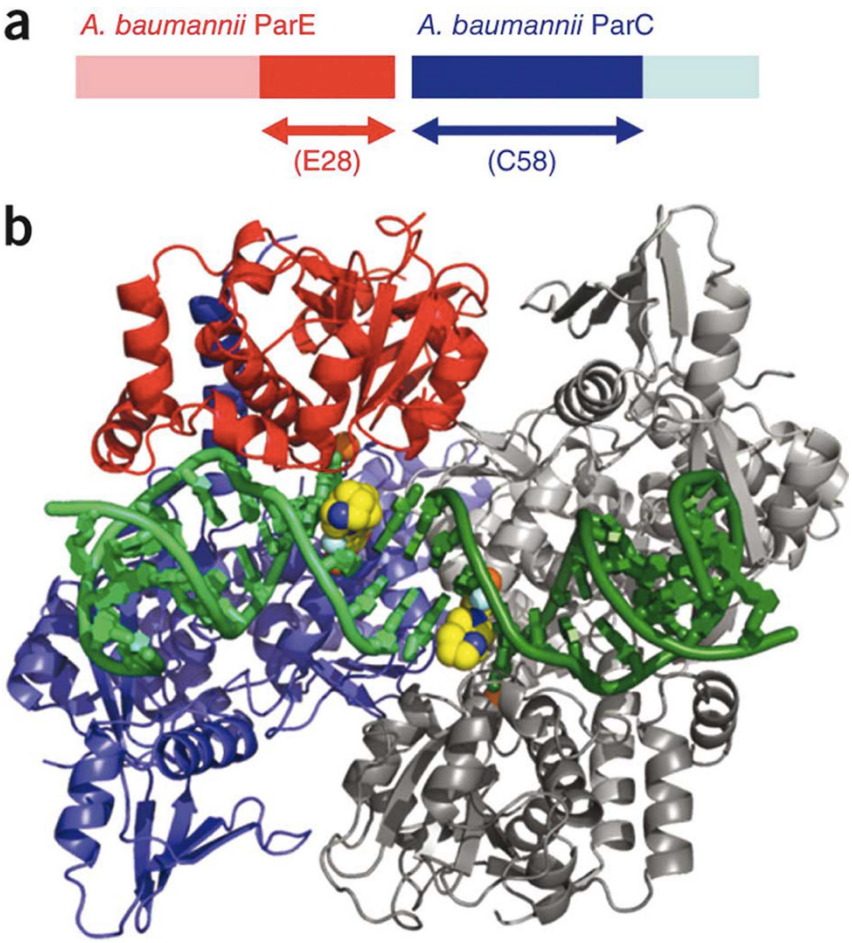


Figure I.11. Crystal structure of *Acinetobacter baumannii* topoisomerase IV in complex with moxifloxacin and DNA (Wohlkonig et al., 2010). (a) C-terminal of ParE region is fused with the N-terminal of ParC. (b) The complex of ParE28-ParC58 with DNA (green), Moxifloxacin (yellow carbons) and magnesium ions (orange spheres).

The development of fluoroquinolone-resistant bacteria has been a cause of major public health concern. Hence, there is a need for identification of novel inhibitors or novel

drug targets. Bacterial topoisomerase I, an essential enzyme in pathogens like *M. tuberculosis*, is being pursued for its potential as a novel drug target (Tse-Dinh 2007, 2009, 2015; Nagaraja, 2017). Bacterial topoisomerases I, a type IA topoisomerase, can form a transient covalent complex during the DNA cleavage-religation reaction of DNA relaxation process. The potential for topoisomerase I mediated accumulation of covalent complexes in the initiation of cell death pathway was studied by random mutagenesis of recombinant topoisomerase I. The mutations on topoisomerase I that can induce the SOS DNA repair system in response to the DNA damage were identified (Cheng et al., 2005; Tse-Dinh, 2009). The studies have highlighted the importance of divalent Mg^{+2} in the DNA rejoining activity of *E. coli* topoisomerase I. The DxD motif in the TOPRIM domain of *E. coli* topoisomerase I is involved in binding with the divalent Mg^{+2}. According to the studies, the D111N mutation of the first aspartate of DxD motif was extremely lethal. The absence of a negatively charged carboxylate side chain of aspartate can be attributed to the loss of Mg^{+2} binding (Cheng et al., 2009). The lack of Mg^{+2} bound to topoisomerase I can inhibit the DNA rejoining after cleavage which can initiate the cell death. Based on these studies, it is ideal to develop small molecules that can serve as poison inhibitors by interfering with the Mg^{+2} binding at the site of topoisomerase I (Tse-Dinh, 2009). It should be noted that a strictly conserved arginine residue in the proximity of the active site is also required for the rejoining activity (Narula et al., 2011). Recently identified bacterial topoisomerases I inhibitors include some phenanthrene compounds (Cheng et al., 2007; Ferrandiz et al., 2014), and Bisbenzimidazoles (Nimesh et al., 2014; Bansal et al., 2010; Ranjan et al., 2014).

Table I. T2. Inhibitors of topoisomerases

Drug	Target	Application
Quinolones (e.g. Nalidixic acid, and Ciprofloxacin)	DNA gyrase, and topoisomerase IV	Antibacterial
Coumarins (e.g. Novobiocin, and Coumermycin A_1)	DNA gyrase, and topoisomerase IV	Antibacterial
Camptothecin (e.g. Topotecan)	Human topoisomerase I	Anticancer
Acridines (e.g. Amsacrine (mAMSA))	Human topoisomerase II	Anticancer
Ellipticines (e.g. 2-methyl-9-hydroxy-ellipticinium acetate)	Human topoisomerase II	Anticancer
Epipodophyllotoxins (e.g. Teniposide)	Human topoisomerase II	Anticancer

C. PROTEIN-PROTEIN INTERACTIONS; CROSS-TALK BETWEEN PROTEINS

An interplay between proteins, protein-protein interactions (PPIs), is required for virtually every cellular process; be it DNA replication, transcription, translation, splicing, secretion, cell cycle control, signal transduction or intermediary metabolism (Phizicky et al., 1995). PPIs are generally transient, and the proteins involved in transient interactions can exist independently. A trigger (for example, a post-translational modification) can initiate interactions between proteins to carry out the essential cellular processes (Perkins et al., 2010). A closer look at the protein-protein interaction network can give us an insight into the multiple roles of individual proteins. Most of the proteins have a unique activity, and the coordination between proteins with unique activities is required for carrying out essential cellular processes. Some proteins could be in a dormant state, until they bind to a regulatory protein partner. Specific domains in the protein will help in the interactions with other proteins, and the binding phenomena could be explained by the hydrophobic bonding, van der Waals forces, formation of a salt bridge (Xu et al., 1997). The strength of binding is sometimes proportional to the size or nature of the binding domain. The interactions with binding partners could be strong or weak, transient or stable (Golemis, 2002).

Protein-protein interactions can result in altered enzyme kinetics as a result of allosteric effects. The protein interactions could up-regulate or down-regulate the activities of proteins involved, implying an inter-dependence of the binding partners. Most of the protein-protein interactions are transient; meaning the proteins part ways, after the desired function is served. The protein oligomers formed by transient interactions can be weak, or

strong in nature (Figure I.12). Weak transient interactions can result in a multitude of dynamic oligomeric states in vivo, whereas a change in the quaternary states of the stronger transient complexes can be triggered by ligand binding.

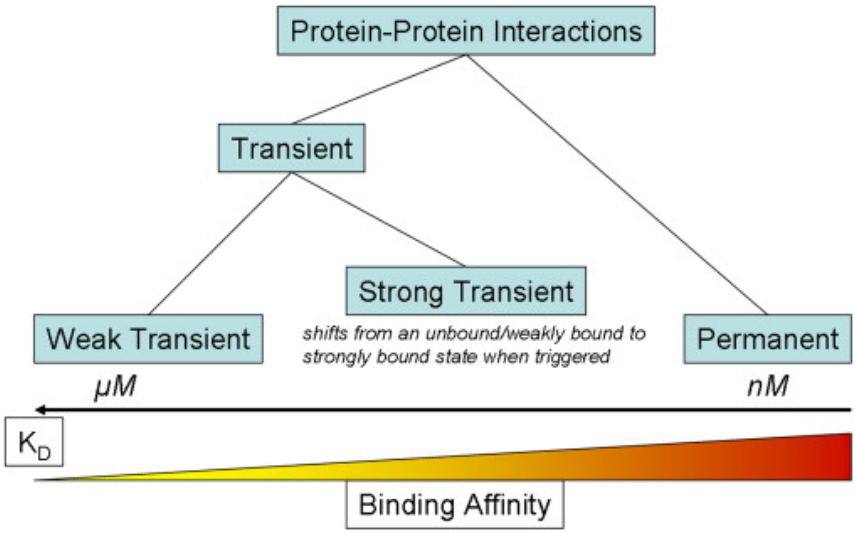


Figure I. 12. Different types of PPIs based on their binding affinities
(Perkins et al., 2010)

It is a challenge to identify the transient PPIs, as the prerequisite for such a study is the identification of the conditions favoring the transient interactions. Some of the widely-used methods for the identification of the PPIs include protein affinity chromatography (pull-down assays), affinity blotting, co-immunoprecipitation (Co-IP), Yeast 2-hybrid system, and far western blot analysis (Edmondson et al., 2001). Pull-down assays, and Co-IP assays can detect strong/stable protein interactions. The coupling of mass spectrometry with the pull-down and Co-IP assays is a powerful tool in the identification of protein complexes (Bymora et al., 2004; Free et al., 2009). Transient/weak interactions can be identified by crosslinking interaction analysis (Yakovlev, 2009), and label transfer protein interaction analysis (Minami et al., 2000). The binding kinetics can be studied by

approaches like surface plasmon resonance (Jonsson et al., 1991), and isothermal calorimetry (Jayanthi et al., 2015). Computational methods to predict the protein-protein interaction networks have gained momentum. However, it is essential to validate the predicted protein-protein interactions through experiments. A commonly used tool, STRING, integrates the known (experimental) and predicted interaction network of protein for a more comprehensive viewing of the protein-protein interaction network (Figure I.13) (Von et al., 2005). The STRING interactions are mapped based on the experimental data, curated databases (text mining), co-expression, protein homology, gene neighborhood, gene co-occurrence, and gene fusion.

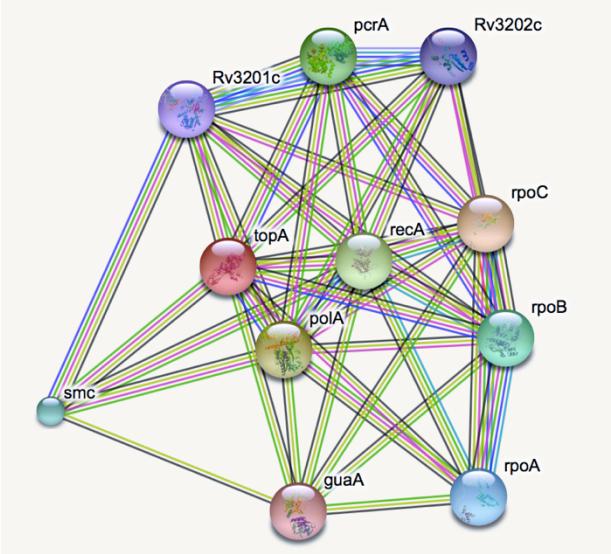


Figure I.13. Results from a STRING search of topoisomerase I (*M. tuberculosis* H37Rv); The protein interaction map of topoisomerase I (topA, red node) is obtained from the STRING search. The search also scores the individual interactions for a measure of confidence. The scores revealed a medium to high confidence in the interactions predicted for topA. (The scores are not shown here) (STRING version 10.5).

Literature has provided evidence of the physical and functional interactions of topoisomerases with its protein partners. Identification of the protein partners of

topoisomerases can give us a valuable insight into the workings of these enzymes. For instance, sporulation and chromosome segregation in *Streptomyces coelicolor*, is only made possible by the recruitment of the Topoisomerase I (TopA) to ParB complexes on DNA. TopA and ParB, in combination, are required for sporulation and it was observed that the depletion of TopA in *S. coelicolor* has inhibited sporulation (Szafran et al., 2013). Human ADP ribosylation factor (ARF) is a recently discovered binding partner of Topoisomerase I, and is a critical component of the cellular reactions that require Topoisomerase I (Karayan et al., 2001). Interactions between RNA recognition motif (RRM) proteins and the cap region of the human topoisomerase I have proved to be critical for the kinase reactions catalyzed by topoisomerase I (Trzcinska-Daneluti et al., 2007). The relaxation activity of the topoisomerase I is inhibited by the binding of the splicing protein, SF2/ASF to the cap region (Amino acids: 215-433) of the topoisomerase I. The cap region of topoisomerase I is attracted to the two closely spaced RRM domains (Amino acids: 1-94) on the SF2/ASF proteins (Kowalska-Loth et al., 2005; Trzcinska-Daneluti et al., 2007). Proteomic analysis of protein partners of human topoisomerase I by Co-immunoprecipitation and affinity chromatography coupled with mass spectrometry had suggested that the N-terminal domain and the cap region of the topoisomerase I have a greater affinity for the proteins in nuclear extracts of HeLa cells. It is important to note that the majority of proteins with an affinity for the cap region of human topoisomerase I enclose two closely spaced RRM domains (Czubaty et al., 2005). Topoisomerase I relaxation activity is enhanced by interactions with ARF (Karayan et al., 2001), CK2, HMG, p53, PARP-1, PSF/P54^{nrp} and Werner protein. In contrast, poly [ADP-ribosylated] PARP-1 and Tax HTLV-1 binding can inhibit the relaxation activity of topoisomerase I

(Czubaty et al., 2005). Binding assays (in vitro), and co-immunoprecipitation assays (in vivo) have provided proof of the specific interactions between *Coprinus cinerus* DNA topoisomerase II (CcTopII) and a meiosis-specific RecA-like protein, Lim15/Dmc1. The Lim15 interaction can potentially activate the in vitro catenation/relaxation activity of CcTopII, and CcTopII down regulates the CcLim15-dependent strand passage activity (Iwabata et al., 2005).

All type IA topoisomerases modify DNA topology by an "enzyme-bridged strand passage mechanism" (Brown et al., 1979,1981). Sequence analysis has indicated that all the type IA topoisomerases are composed of a core, with conserved motifs and an active site (Berger, 1998; Champoux, 2001; Corbett et al., 2004), and a carboxy-terminal region, highly variable in size and sequence, containing a variable number of Zn^{+2} ribbon motifs ranging between 1 and 5 (Tse-Dinh et al., 1988; Bhaduri et al., 1998; Serre et al., 2003; and Bouthier et al., 1998). The C-terminal domains of type IA topoisomerase have been implicated in protein interactions that define the physiological activity of a topoisomerase (Figure I.14) (Viard et al., 2007). In humans, type IA topoisomerases have two representatives: Topoisomerase IIIα (TopIIIα) and Topoisomerase IIIβ (TopIIIβ) (Wang, 2002). The structural gene of TopIIIα is located on chromosome 17p11.2-12 (Hanai et al., 1996) and the gene for TopIIIβ, a variant of TopIIIα is mapped within Ig lambda locus on chromosome 22q11-12 (Kawasaki et al., 1997). The existence of two different enzymes within the eukaryotic type IA topoisomerase sub-family could be justified by their functional differences through association with different helicases (Li et al., 1998). Experiments have been conducted to test the significance of these enzymes by targeted gene-disruption experiments in mice. The inactivation of TopIIIα in mice resulted in

embryonic lethality shortly after implantation (Li et al., 1998). A similar disruption of the TopIIIβ gene had no apparent effect on the embryo, but a reduction in the fertility accompanied by aneuploidy and reduced life span was observed (Kwan et al., 2001; Kwan et al., 2003). According to recent reports, deletion of TopIIIβ can contribute to neurodevelopmental disorders (Stoll et al., 2013), cognitive impairment, and facial dysmorphism (Kaufman et al., 2016). TopIIIβ is a RNA topoisomerase that has been linked to schizophrenia and autism. The physical and functional association of human TopIIIβ with fragile X mental retardation protein (FMRP) is required for the regulation of the translation of mRNAs that are essential for the neuronal functions. Mutation in the FMRP can abolish the interaction with TopIIIβ, and can contribute to the development of neuronal disorders (Xu et al., 2013).

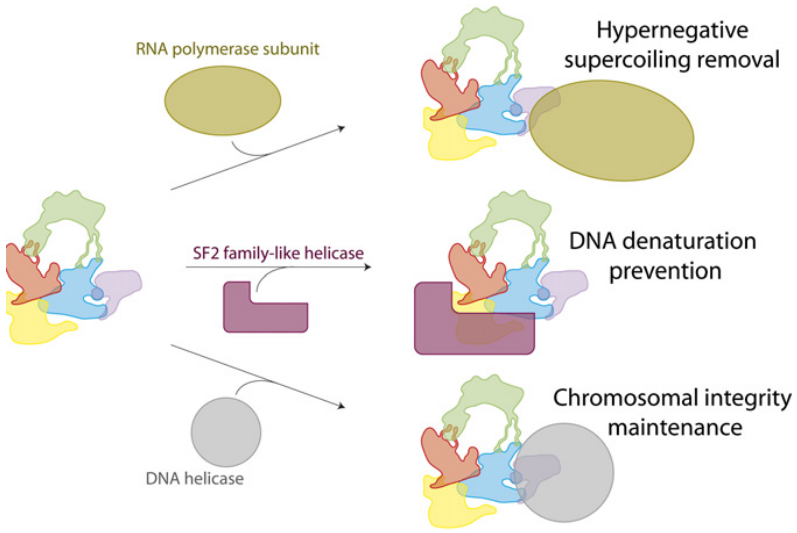


Figure I.14. Schematic structures of known toposomes with their in vivo roles (Viard et al., 2007)

The determinants of Bloom's syndrome and Werner's syndrome, the BLM and WRN genes respectively, encode for proteins resembling budding yeast SGS1 gene

product (Ellis et al., 1995, Yu et al., 1996), a DNA helicase that interacts physically and functionally with topoisomerase III (Gangloff et al., 1994 and Lu et al., 1996). Bloom's syndrome, and Werner's syndrome are associated with high incidence of cancers (Ellis et al., 1995, Yu et al., 1996). TopIIIα is recruited by the helicase BLM to resolve the in vivo non-canonical DNA structures (Double holliday junctions, D-loops, G-quadruplexes) (Wu et al., 2003). In Bloom syndrome patients, BLM helicase is mutated and fails to recruit TopIIIα, thereby increasing the sister chromatid exchanges in Bloom syndrome cells. Bloom syndrome patients are often diagnosed with cancer in their mid-twenties (German, 1997). Gaining an understanding of the factors favoring or inhibiting the protein-protein interactions could be a huge advance in our understanding of the disease, and therapy.

A paradigm shift is happening in the field of topoisomerases. The focus has been shifted from understanding the role of topoisomerases as independent units to the complexes they form with other proteins. The in vivo role of a topoisomerase is defined by the protein partner it binds, rather than being encoded in its sequence or structure (Viard et al., 2007). The term "toposome" has been coined for the protein complexes formed with the C-terminal domains of topoisomerase (Borowiec, 2004; Lee et al., 2004). There is increasing evidence surfacing to support the view that toposomes, and not independent units of topoisomerases, are involved in the physiological functions (Viard et al., 2007). Bacterial topoisomerase I, the subject of my research, belongs to the type IA subfamily. It should be noted that my research is aimed at the study of protein-protein interactions of topoisomerase I from *Escherichia coli, Mycobacterium smegmatis*, and *Mycobacterium tuberculosis*.

D. BACTERIAL TOPOISOMERASE I

Bacterial topoisomerase I, a type IA topoisomerase of bacteria, can relax negatively supercoiled DNA through an enzyme-bridged strand passage mechanism (Figure I.15). The enzyme can form a transient 5' covalent intermediate during the relaxation process. The bacterial topoisomerase I does not require an external source of energy, but requires a divalent cation for its activity (Baker et al., 2009).

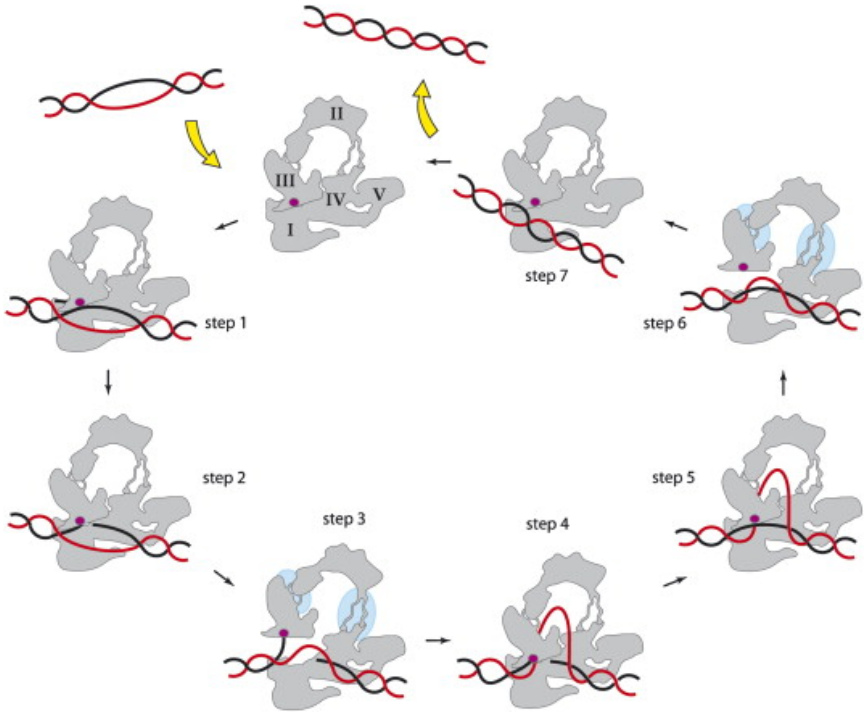


Figure I.15. Proposed enzyme bridged strand passage mechanism of relaxation by bacterial topoisomerase I (Viard et al., 2007). Step 1: Active site, tyrosine, binds to the substrate; Step 2: Cleavage of the substrate; Step 3: A change in Conformation of the enzyme, resulting in an opening of the gate. Step 4: Transfer of the unbound strand, and gate closure; Step 5: Resealing of the nicked substrate (Religation); Step 6: Change in the conformation of the enzyme, favoring the release of the relaxed substrate; Step 7: Release of the relaxed substrate, and return of the enzyme to its original conformation.

Across bacteria, the type I topoisomerases, are similar in their structure, sequence, and mechanism of action. Although the N-terminal domains D1-D4 of topoisomerase I

share a high level of sequence homology, the C-terminal domains are highly variable in its size and sequence. It is believed that the C-terminal domains have a significant role in the DNA or protein interactions (Zhang et al., 1994; Beran-Steed et al., 1989). Bacterial topoisomerase I is present in all bacteria. However, bacterial topoisomerase III, another representative of the type IA topoisomerase family, is present only among some of the bacterial species. For instance, *M. tuberculosis* has only one representative of the type IA topoisomerase in the form of topoisomerase I. However, topoisomerase I and topoisomerase III are both present in *E. coli*. Studies have revealed the indispensability of the topoisomerase I in *M. tuberculosis*, validating its potential as a drug target in this pathogen (Godbole et al., 2015; Nagaraja et al., 2017; Ravishankar et al., 2015). Transposon insertion or deletion mutants of the *E. coli topA* gene (encoding topoisomerase I) were successfully isolated. However, attempts to isolate insertion or deletion mutants of the *topA* gene in *M. tuberculosis* or *Helicobacter pylori* were unsuccessful (Sassetti et al., 2003; Suerbaum et al., 1998). Recent reports have confirmed that the silencing of the *topA* gene significantly affected the survival of *M. tuberculosis* (Ravishankar et al., 2015, Ahmed et al., 2014).

***E. coli* topoisomerase I (EcTopoI)** is involved in the removal of transcription-driven negative supercoiling behind the RNA polymerase complex (Liu et al., 1987; Masse et al., 1999a). A loss of topoisomerase I function can possibly lead to the accumulation of R-loops (RNA-DNA hybrids) along with an increased torsional stress. A simultaneous depletion of the RNAse HI (an R-loop hydrolyzing enzyme) and topoisomerase I can have a severe effect on the viability due to interference in the transcription and replication processes by R-loop accumulation (Stockum et al., 2012). Although the active site is

located on the N-terminal domains D1-D4 of EcTopoI, the N-terminal fragment of D1-D4 cannot catalyze the removal of the negative supercoiling by itself. The enzyme requires the C-terminal domains, along with the N-terminal domains D1-D4 for its activity (Terekhova et al., 2013). The C-terminal region has three zinc ribbon domains D5-D7 that can hold three zinc(II) ions and two zinc ribbon like domains D8-D9 (Tse-Dinh et al., 1988, Tan et al., 2015). Mutating the cysteine residues on the zinc ribbon motifs, or removal of the Zinc(II) rendered the enzyme inactive (Tse-Dinh, 1991; Zhu et al., 1995).

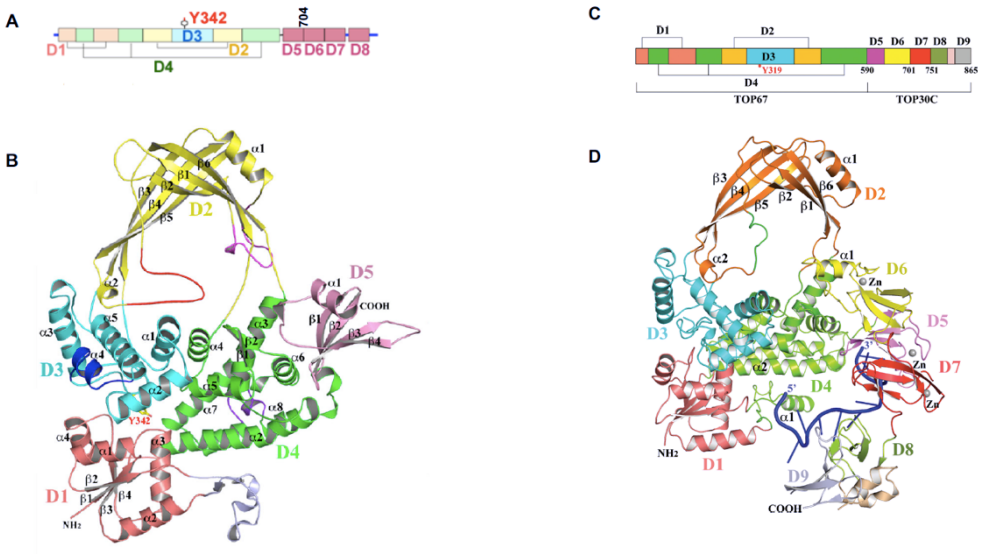


Figure I.16. Crystal structures of bacterial topoisomerase I; (A) Domain arrangement of *M. tuberculosis* topoisomerase I (MtbTopoI) (B) Crystal structure of MtbTopoI-704t (D1-D5) (Tan et al., 2016) (C) Domain arrangement of *E. coli* topoisomerase I (EcTopoI) (D) Crystal structure of full-length EcTopoI in complex with single stranded DNA (Tan et al., 2015).

The most recent crystal structure of the full-length enzyme in coordination with a single stranded DNA has provided evidence for the role of the C-terminal domains D5-D9 in recognition and interaction with the hyper-negatively supercoiled DNA (Figure I.16 (D))

(Tan et al., 2015). The enzyme utilizes both the N-terminal and C-terminal domains for the interactions with the hyper-negatively supercoiled DNA in relieving the supercoiling stress. Briefly, the N-terminal domains recognize and cleave a strand of DNA (Gate-strand) via its active site, whereas the zinc ribbon domains D5-D7 in the C-terminal region can bind the transfer-strand of the under-wound substrate, allowing the passage of the transfer strand through the gate (Figure I.15). The role of the C-terminal is not just limited to the binding under wound DNA, as literature has suggested its role in the protein-protein interactions. A direct interaction of the C-terminal region of EcTopoI with the RNA polymerase β' subunit can ensure a smooth transcription elongation (Cheng et al., 2003).

The topoisomerase I of *M. tuberculosis* (MtbTopoI) has been investigated for its potential as a novel drug target in the treatment of drug resistant tuberculosis. Unlike in *E. coli*, the pathogen *M. tuberculosis* has only one representative of type I (MtbTopoI), and type II topoisomerase (gyrase). The MtbTopoI relaxes the topological strain on the DNA by cutting and religating a single-strand of DNA, much like *E. coli* topoisomerase I. Genetic studies have provided an evidence for the indispensability of the only type I topoisomerase (MtbTopoI) in *M. tuberculosis* (Ahmed et al., 2014). *M. smegmatis*, a non-pathogenic mycobacterium, also has only one type IA topoisomerase (MsmTopoI). Genetic studies on the topoisomerase I-depleted strain has provided evidence for the essentiality of MsmTopoI in the growth and nucleoid architecture (Ahmed et al., 2015). Mycobacterial topoisomerase I is devoid of the Zinc-binding tetra-cysteine motifs, unlike the *E. coli* topoisomerase I. The N-terminal domains of topoisomerase I are highly conserved among different bacteria, whereas the C-terminal domains are highly variable. A crystal structure of the MtbTopoI-704t (N-terminal domains: D1-D4, and C-terminal domain: D5) was

recently published (Figure I.16 (B)) (Tan et al., 2016). The MtbTopoI-704t was sufficient for the DNA cleavage-religation activity, and single stranded DNA catenation but not for relaxation of negatively supercoiled DNA (Tan et al., 2016). The C-terminal region of mycobacteria and other actinobacteria are divergent from the other bacterial species, and contains three basic stretches that are required for binding with the non-scissile strand of DNA, allowing the strand passage through the cleaved strand (Ahmed et al., 2013). Recent, X-ray crystallography structural studies showed that the *Mycobacterium tuberculosis* topoisomerase I C-terminal domains (CTD) are formed by repeats of a novel protein fold of a four-stranded antiparallel β-sheet stabilized by a crossing-over α-helix (Tan et al., 2016).

OVERVIEW

The topological state of DNA is managed by a group of enzymes called topoisomerases. Essential cellular processes like transcription, replication, recombination and DNA repair create topological problems for DNA. There is a consensus in the scientific community about the role of topoisomerases in addressing the topological problems of DNA generated during the above-mentioned processes. The indispensability of these enzymes for the viability of a cell have made them the potential targets of antibiotic and anticancer drugs. However, the potential for success depends on how well we understand the in vivo behavior of topoisomerases. The goal of my research is to understand the functional association network of bacterial topoisomerase I by studying its protein-protein interactions. In chapter 1, the molecular mechanism of interaction between *E. coli* RNA polymerase and topoisomerase I was elucidated. The protein interaction between RNA polymerase beta'

subunit and topoisomerase I of *E. coli* was previously reported, and the association is believed to resolve the transcription driven hyper-negative supercoiling. The key amino acid residues of topoisomerase I and RNA polymerase favoring the interaction are predicted by molecular docking, and molecular dynamic simulations. The effect of mutating some of the key topoisomerase I residues on the interaction between RNA polymerase and topoisomerase I was also reported. In chapter 2, the protein-protein interactions of *M. smegmatis* topoisomerase I were analyzed by pull-down and co-immunoprecipitation assays (Co-IP) coupled to mass spectrometry. The interaction of RNA polymerase with topoisomerase I in *M. smegmatis* was established by both pull-down, and Co-IP. In chapter 3, the mechanism of interaction and the physiological relevance of the RNAP-topoisomerase I interaction in *M. smegmatis* was explored. A novel mechanism of interaction through the C-terminal tail of topoisomerase I was reported. In chapter 4, the molecular basis for stimulation of *E. coli* topoisomerase I catalytic activity by RecA was investigated. To study physical interactions as the basis of stimulation of topoisomerase I activity by RecA, the physical interactions were examined by molecular docking, pull-down assays, and surface plasmon resonance. The experimental evidence supported a direct physical interaction of *E. coli* RecA with the N-terminal domains of topoisomerase I.

CHAPTER 1

MOLECULAR MECHANISMS OF INTERACTION BETWEEN *E. COLI* RNA POLYMERASE AND TOPOISOMERASE I

ABSTRACT

Escherichia coli topoisomerase I (EcTopoI), a type IA DNA topoisomerase, physically and functionally interacts with RNA polymerase (RNAP) during the transcription process. EcTopoI, an enzyme responsible for relieving the negative supercoiling, is recruited by RNAP beta' subunit for resolving the transcription associated hyper-negative supercoiling. Structural information of the RNAP-EcTopoI complex is required for understanding the basis of protein-protein interaction. The potential structure of the complex was predicted by molecular docking, and molecular dynamic simulations. Surface plasmon resonance (SPR) provided a quantitative measure of the interaction (K_D=~93 nM). The docking studies predicted salt bridging and hydrogen bonding as the driving force for interaction of the RNA Polymerase beta' subunit with the C-terminal domain of topoisomerase I in *E. coli*. Residues 609 (Arginine), 627 (Lysine), and 664 (Lysine) of EcTopoI were predicted, by docking, to be involved in the salt bridge and hydrogen bond formation with RNAP beta' subunit. Supporting the docking prediction, pull-down assays with a double mutant EcTopoI (K627,664A) created in this study via site-directed mutagenesis confirmed a significant reduction in the interaction with RNA polymerase.

INTRODUCTION

Transcription initiation, elongation, and termination is driven by DNA-dependent RNA polymerase (RNAP) across domains of life (Ray-Soni et al., 2016; Vassylyev et al, 2007; Feklistov, 2013; Vassylyev, 2009). The core subunits of RNAP share a high degree of homology in prokaryotes and eukaryotes (Sweetser et al., 1987; Cramer, 2002, Ebright, 2000). The progression of the RNAP complex along the DNA template during transcription elongation can create hyper-negative supercoiling behind the complex, and Positive supercoils ahead of it (Liu et al.,1987; Vos et al., 2011; Ma et al., 2013). The supercoiling problem needs to be addressed for an unhindered transcription process, and topoisomerases are involved in relieving such tension on the DNA (Wu et al., 1995).

Other essential cellular processes including replication, repair, and recombination also require the activities of topoisomerases for genomic stability. Bacterial topoisomerase I, a type IA DNA topoisomerase, relaxes negative supercoils in DNA (Champoux, 2002a). *Escherichia coli* topoisomerase I (EcTopoI), the most widely studied bacterial type IA topoisomerase, consists of 865 amino acid residues organized into N-terminal domains D1-D4 and C-terminal domains D5-D9 (Figure I.16, C). EcTopoI can bring about the relaxation of negatively supercoiled DNA by forming a transient 5' phosphotyrosine linkage with a single strand of DNA through its active site residue-Y319 (Lynn & Wang, 1989). R-loop formation and a friction in the transcription elongation process can occur in the absence of EcTopoI activity (Drolet et al., 1995; Masse et al., 1999b). The functional association of RNAP-EcTopoI may require a physical association of these proteins. Previous reports have verified a direct physical interaction of the C-terminal domain of topoisomerase I (EcTopoI-CTD) with the RNAP beta' subunit (Cheng et al., 2003).

However, information regarding the structure of the protein-protein interaction complex and molecular mechanism of the interaction remained to be elucidated. In silico molecular docking, and molecular dynamic simulations were performed for predicting the binding modes, and identification of the amino acid residues involved in the complex formation. Prediction of the structure of a protein-protein complex (Smith et al., 2002; Gray et al., 2003) and binding interface (Xue et al., 2015) has been made possible by molecular docking. The affinity and strength of a biomolecular interaction can be studied by a widely-used label free technique, surface plasmon resonance (SPR) (Tiwari et al., 2014; Elass et al., 2007; Tiwari et al., 2015). Equilibrium dissociation constant (K_D), a parameter for measuring the binding affinity, can be determined by SPR (Kastritis et al., 2013). SPR was used for the quantitative characterization of the interaction between RNAP and EcTopoI.

The conformational dynamics of proteins as flexible macromolecules can be related to its function (Karplus et al., 2002). In silico studies can provide insights into the influence of a protein conformation on its binding partner and the strength of binding (Singh et al., 2016). The structural features of RNAP-EcTopoI complex were modelled by all-atom molecular dynamic simulations of the complex predicted by molecular docking. The docking studies, and all-atom molecular dynamic simulations complemented the experimental evidence for interaction between RNAP beta' subunit and the C-terminal domain of EcTopoI (Cheng et al., 2003). The RNAP-EcTopoI complex was stable for entirety, 150 nanoseconds, of simulation. Stable hydrogen bonds are established between S1117 (RNAP) and K664 (EcTopoI), V697 (RNAP) and K664 (EcTopoI). As the complex establishes further inter-protein interactions, the RNAP β' subunit undergoes a significant structural rearrangement favoring the salt bridge formation between E874 (RNAP) and

K627 (EcTopoI), E1009 (RNAP) and R609 (EcTopoI). It is possible that the hydrogen bonding initiates the EcTopoI-RNAP complex formation, and supports the structural flexibility of the RNAP β' subunit without breaking the complex. In this study, the lysine residues predicted to be essential for hydrogen bonding and salt bridge formation were mutated to alanine. The mutant recombinant EcTopoI proteins were expressed and purified. The effect of these site-directed mutations on the complex formation with RNAP was verified by pull-down assays.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli strain BW25113 (*Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ⁻*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*), obtained from Yale CGSC (Datsenko and Wanner, 2000), was used for preparing the cell lysate used in the pull-down of RNAP from total soluble cellular proteins. Plasmid, pLIC-ETOP transformed into *E. coli* BL21-AI was used for the expression and purification of recombinant *E. coli* topoisomerase I with 6x-His tag (Sorokin et al., 2008). The mutant plasmids (pLIC-ETOP(K627A), pLIC-ETOP(K664A), pLIC-ETOP (K627,664A)) were constructed by site directed mutagenesis of pLIC-ETOP. The plasmids were transformed separately into *E. coli* BL21 STAR DE3 (Invitrogen) for overexpression, and purification of the mutant EcTopoI. A pET His_6-Mocr TEV cloning vector (2O-T) (gift of Scott Gradia, Addgene #29710) was used for expression and purification of a recombinant viral protein, His-Mocr (DelProposto et al., 2009) that was used as negative control in the pull-down assays.

Table 1. T1. Primers used in Site-Directed Mutagenesis

Primer	Sequence (5'-3')	Description
pLIC-ETOP_K627A_FP	GCTGCCGCCGGCAGAGCGTTGCA	Lysine 627 of EcTopoI is mutated to Alanine
pLIC-ETOP_K627A_RP	GCATAGCCAGAACAGCCAAGG	
pLIC-ETOP_K664A_FP	TCGTTGCCCGGCATGCGGCACGG	Lysine 664 of EcTopoI is mutated to Alanine
pLIC-ETOP_K664A_RP	CGTTTTGCGCGCAGCGCG	
pLIC-ETOP_K627,664A_FP	GCTGCCGCCGGCAGAGCGTTGCA, TCGTTGCCCGGCATGCGGCACGG	Lysine 627 and 664 of EcTopoI is mutated to Alanine (Double mutant)
pLIC-ETOP_K627,664A_RP	GCATAGCCAGAACAGCCAAGG, CGTTTTGCGCGCAGCGCG	

Protein purification

E. coli topoisomerase I with a N-terminus 6x-Histidine tag (His-EcTopoI) was expressed from pLIC-ETOP in *E. coli* BL21-AI by induction with 1 mM IPTG, 0.02% L-Arabinose as described previously (Sorokin et al., 2008). Mutant *E. coli* topoisomerase I with a N-terminus 6x-Histidine tag was expressed from a mutant pLIC-ETOP plasmid in BL21 Star (DE3) by induction with 1 mM IPTG. Expression of recombinant His-tagged Mocr was induced in BL21 star (DE3) with 1 mM IPTG. Protein purification was carried out with a PrepEase Histidine-tagged protein purification maxi kit (Affymetrix; 78805), according to the recommended manufacturer's protocol.

Relaxation assay

The in vitro relaxation activity of the purified mutant EcTopoI enzyme was compared with the wild-type enzyme. A supercoiled pBAD/Thio plasmid DNA substrate (160 ng), purified by Cesium Chloride gradient method, was incubated with different amounts of the enzyme in the presence of 5 mM $MgCl_2$ (Sandhaus et al., 2016). The reaction mixture was setup at 37°C for 30 minutes. The reaction was terminated by the addition of stop buffer (50 mM EDTA, 50% glycerol, 0.5% v/v bromophenol blue), and the reaction products were electrophoresed on a 1% agarose gel and then stained with ethidium bromide for visualization of the DNA.

Molecular docking

The structure of the *E. coli* RNAP core enzyme was taken from protein data bank (PDB) [PDB entry 3LU0 (Opalka et al., 2010)]. Protein coordinates in the PDB entry 4RUL

(Tan et al., 2015) was taken as the full-length structure of EcTopoI. Molecular docking between these two proteins was performed by Dr. Tiwari using the PatchDock web server (Duhovny et al., 2002 & 2005). The top 100 predictions from the PatchDock docked complexes were refined using FireDock web server (Mashiach et al., 2008; Andrusier et al., 2007). The best-ranked complex with the lowest energy (highest affinity) from the FireDock output was considered as the RNAP-EcTopoI complex for further analysis.

Surface plasmon resonance (SPR)

Biacore T200 SPR instrument was used to determine the K_D value for RNAP-EcTopoI complex formation. Recombinant EcTopoI (ligand) expressed and purified, as described previously (Sorokin et al., 2008), was immobilized onto series S CM5 sensor chip (GE Healthcare) via standard amine coupling chemistry. Various concentrations of RNAP core enzyme (New England BioLabs) were used as analyte to flow (in duplicates or triplicates) over the ligand-immobilized surface at a flow rate of 50 µL/min. 20 mM Tris-HCl (pH7.5) supplemented with 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 0.005% Tween-20 and 5% glycerol was used in the RNAP-EcTopoI SPR binding experiments. 1M NaCl was used to regenerate the sensor surface. SPR experiments were carried out at 25^{o}C.

Molecular dynamics (MD) Simulations

All-atom MD simulations were carried out with the NAMD simulation package (Phillips et al., 2005). The interacting domains of RNAP-EcTopoI complex were considered for the simulation studies. These domains consisted of residues 788-1317 from

the RNAP β' subunit and residues 594-705 from EcTopoI. Two Zn^{2+} ions bound to the tetracysteine motifs are within this selected EcTopoI region (594 to 705). The input files/parameters for MD simulations were generated by CHARMM-GUI (Lee et al., 2016) using CHARMM36 force field. The complex was solvated in a cubic box with TIP3 water and the system was electrically neutralized by adding ten Mg^{2+} and four Cl^- ions. The resulting dimension of the solvation box was 120 x 120 x 120 $Å^3$, containing 164,880 atoms. The MD simulations of the complex started with 10,000 steps of energy minimization with the conjugate gradient and line search algorithm. The Particle Mesh Ewald (PME) (Darden et al., 1993) method was used for the long-range interactions with 12Å non-bonded cut-off. The system was equilibrated at 300 K (27^oC) for 100 ps with protein heavy atoms harmonically restrained at a 1 fs integration time step, followed by a 4-ns NPT run with fully unrestrained protein using a 2 fs time step. The simulation was then propagated with Langevin dynamics with a damping constant of 1 ps^{-1}. The complex was then simulated for 150 ns under NVT condition with a time step of 2 fs.

Data analysis

Biaevaluation software version 2.0 was used to evaluate the SPR sensorgrams and determine the K_D value via steady state affinity analysis. Each frame in the simulation trajectory file was saved at every 20 ps. VMD (Humphrey et al., 1996) was used to visualize the structure and analyze the simulation trajectories.

Pull-down assays on *E. coli* soluble cell lysate

In this assay, *E. coli* strain (BW25113) cultured in LB medium for 16 hours to stationary phase (OD_{600}=2.5), and the culture was pelleted. The cell pellet was suspended in the pull-down buffer containing 1mg/ml lysozyme. The suspended cells were subjected to lysis by four freeze-thaw cycles. The lysate was centrifuged at 13000xg for 2 hours at 4°C. The soluble protein fraction was incubated (precleared) with HisPur Cobalt Agarose resin before its use in the pull-down reaction. The preclearing minimizes non-specific binding of proteins to the beads. Either purified wild type EcTopoI or mutant EcTopoI was used as bait. A Bacteriophage T7 protein, Mocr, with a N-terminus 6x-His tag was used as bait in the negative control for the pull-down assay (DelProposto et al., 2009). Bait (40 nM), and precleared soluble protein fraction (200 μg) were incubated at 4°C for 2 hours. HisPur Cobalt agarose resin (Thermofisher) was added to the reaction, and incubated overnight at 4°C. On the following day, the resin-reaction mixture was spun, and the supernatant was discarded. The bead pellet was washed three times in pull-down buffer with 10 mM imidazole to minimize non-specific binding of histidine rich proteins to the resin. The proteins bound to the resin were finally eluted in 400 mM imidazole, and the eluates were subjected to SDS-PAGE analysis. A western blot was performed to probe for RNA polymerase using a monoclonal antibody against RNA polymerase beta (BioLegend).

RESULTS AND DISCUSSION

Salt bridges and hydrogen bonds drive the complex formation

The molecular docking and molecular dynamic simulations have provided the structural information of the RNAP-EcTopoI complex. The computational models agree with the experimental evidence, as the binding interface predicted by the modeling also involves the RNAP β' subunit and the C-terminal of EcTopoI (Cheng et al., 2003). The structure of the most favorable RNAP-EcTopoI complex, predicted by PatchDock and refined by FireDock is shown in Figure 1.2, A.

Analysis of the RNAP-EcTopoI complex interface (Fig. 1.2, E) after the energy minimization shows that several hydrogen bonds, ionic, and van der Waals interactions are involved in forming and stabilizing the RNAP-EcTopoI complex. The interfacial contacts involve mainly the residues in the EcTopoI loop segments that are stabilized by Zn^{2+} co-ordinations at the two ends. Specifically, inter-protein salt-bridge interactions between E873 (RNAP) and K627 (EcTopoI), and between K959 (RNAP) and E628 (EcTopoI) occur at the interface involving one of the Zn^{2+} bound loop of the EcTopoI-CTD. Other specific interactions include hydrogen bond between S876 (RNAP) and E628 (EcTopoI), and between V967 (RNAP, backbone) and K664 (EcTopoI, side chain). Although these salt-bridge and hydrogen bond interaction pairs change over the course of MD simulation, the residues involved in such interactions represent the recognition sites for EcTopoI-CTD binding to RNAP. Remarkably, the EcTopoI region that binds to RNAP is very close to the location where it binds the single stranded DNA in the crystal structure of the EcTopoI-DNA complex (Figure 1.2, F), making the inter-protein interactions in this region

functionally relevant, as the DNA may be an extension of the RNAP transcription elongation complex.

The stability of the complex was assessed by all-atom MD simulations in an explicit solvent. A significant rearrangement of the interfacial contacts was observed. The final structure the complex at the end of the 150 ns NVT simulation is shown in Figure 1.3, A. The contacts are observed in three different sites, two of which involves residues in the loop segments that are stabilized by the Zn^{2+} co-ordinations at the two ends of EcTopoI. Relatively more stable interfacial contacts are made in the Zn^{2+} coordinated loop involving K664. Specifically, the positively charged K664 (EcTopoI) side chain forms two hydrogen bonds: with the S1117 side chain and the V967 backbone (RNAP β' subunit). Slight structural rearrangements yield two new salt-bridges K627 (EcTopoI)-E874 (RNAP) and R609 (EcTopoI)-E1009 (RNAP) to replace the two originally observed ionic interactions K627 (EcTopoI)-E873 (RNAP) and E628 (EcTopoI)-K959 (RNAP). The salt-bridge interaction between R609 (EcTopoI) and E1009 (RNAP) occurs about midway between the Zn^{2+} coordinated sites. The amino acid residues in EcTopoI that establish salt-bridges with RNAP are generally conserved among various bacterial species (Fig. 1.3, C). Interestingly, when lysine in position 664 is replaced by isoleucine, its binding counterpart serine in position 1117 is also replaced by isoleucine or valine. This makes it possible for these residues to potentially interact via hydrophobic interactions. The K664 of EcTopoI, responsible for a stable hydrogen bonding with RNAP, helps in latching on to RNAP β' subunit and stabilizing the complex.

EcTopoI-RNAP have high binding affinity

SPR analysis was carried out for determining the equilibrium dissociation constant (K_D) of the complex. The steady state response values (shaded grey area in Fig. 1.2, C) were plotted against the RNAP concentration (Fig. 1.2, D) and were evaluated using Biaevaluation software version 2.0 via steady state affinity model fitting. The calculated K_D value for RNAP-EcTopoI complex was ~93 nM, indicating a strong affinity between these proteins.

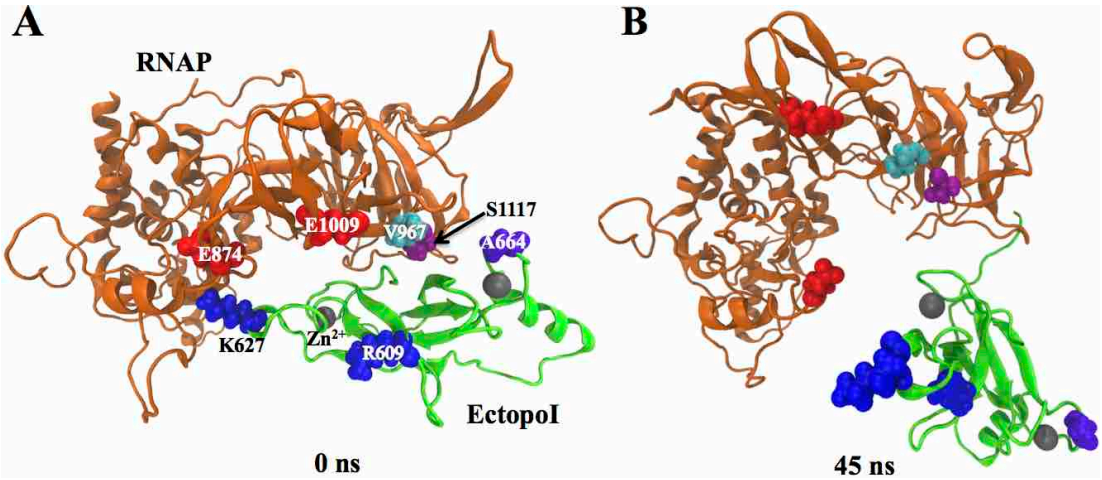


Figure 1.1. MD simulation of mutant EcTopoI (K664A) in complex with RNAP at different times of the simulation trajectory. (A) Structure of the complex at 0 ns (B) Dissociation of the complex at 45 ns.

Salt bridging and hydrogen bonding are essential for the complex formation

Molecular docking and MD simulations have predicted the RNAP-EcTopoI complex formation through salt-bridging, and hydrogen bonding. According to these studies, the K664 plays a significant role in the complex formation and stabilization. The MD simulation studies on the RNAP-EcTopoI complex formation by mutating the K664 of EcTopoI to alanine resulted in the dissociation of the complex in 45 ns (Figure 1.1, B).

The experimental evidence for the significance of lysine residues (K627, K664 of EcTopoI) in RNAP-EcTopoI interactions was verified by Pull-down assays.

The wild type, and mutant EcTopoI (K627A, K664A & K627,664A) with a 6xHis tag on the N-terminus was purified, and used as a bait in the pull-down assays. The relaxation activity of the purified mutant enzymes was no different from the wild type EcTopoI (Figure 1.4, A). Mutating the lysine 627 or 664 of EcTopoI to alanine has caused a reduction in binding with RNAP (Lane 4, 5 of Figure 1.4, B). When both the lysine were mutated to alanine, there was significant reduction in binding with RNAP (Lane 6 of Figure 1.4, B). The diminished binding can be attributed to the inability of the mutated EcTopoI to form salt bridges and hydrogen bonds with RNAP beta' subunit.

CONCLUSIONS

The formation and stabilization of the RNAP-EcTopoI complex using MD simulations was investigated. The SPR measurements suggest a strong binding affinity between RNAP and EcTopoI (K_D= ~93 nM). The RNAP-EcTopoI complex structure was generated by molecular docking, optimized and analyzed with all-atom MD simulations. Consistent with the previous experimental result, the predicted binding interface involves the RNAP β' subunit and EcTopoI-CTD. The complex was stable throughout the 150 ns of MD simulation. The amino acid residues involved in the formation and stabilization of the protein complex were identified. Two salt-bridges between E874 (RNAP) - K627 (EcTopoI), and E1009 (RNAP) - R609 (EcTopoI) as well as two hydrogen bonds between S1117 (RNAP) - K664 (EcTopoI), and V967 (RNAP) - K664 (EcTopoI) comprise the major interfacial interactions in the RNAP-EcTopoI complex. Upon EcTopoI binding,

RNAP was found to undergo conformational rearrangements, facilitating the inter-protein interactions. The mutant complex with the K664A substitution is found to be unstable, according to the simulation studies. The removal of the specific interactions involving K664 and S1117 allows the complex to dissociate, with no inter-protein contacts left after 40 ns of simulation. However, according to the pull-down experiments, mutating the EcTopoI K664 alone did not hinder the association with RNAP, although a reduction in the binding was noticed. There was a significant effect on the complex formation, when both the lysine (627, and 664) were mutated to alanine. Based on these observations, it can be speculated that the EcTopoI loop, containing K627 and K664, is responsible for the initiation of the complex formation. Since this loop is stabilized by Zn^{2+} coordination, it is possible that the Zn^{2+} coordination might also be important for the complex formation. It will be interesting to study the in vivo response of bacteria, when the amino acids residues critical for RNAP-EcTopoI complex formation are mutated. The RNAP-topoisomerase I interactions are essential for relieving the transcription driven hyper-negative supercoiling, and it is possible that this association is conserved across different life forms. In chapter 3, a novel mechanism of RNAP-Topoisomerase I interaction in mycobacteria is discussed.

Acknowledgments

Molecular docking, simulation studies and SPR experiments were carried out by Purushottam Babu Tiwari (Georgetown University). The computational and SPR work discussed in this chapter is published in FEBS Letters (Tiwari et al., 2016).

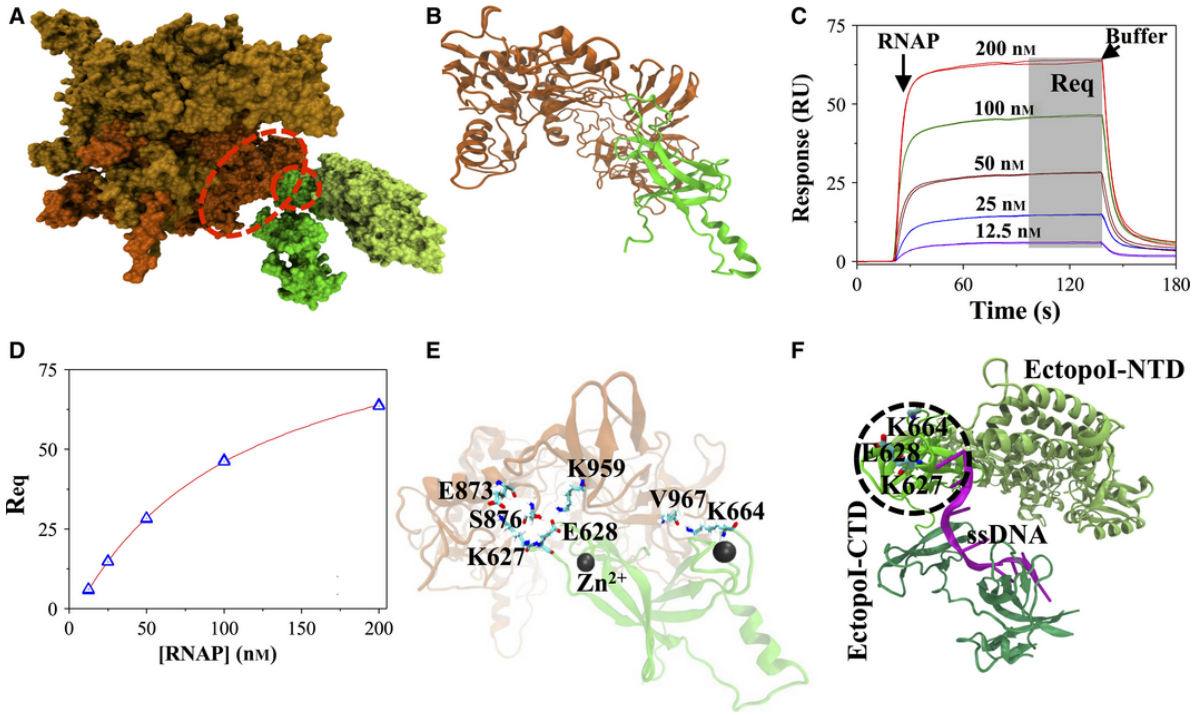


Figure 1.2. Association of RNAP-EcTopoI (Tiwari et al., 2016). (A) The complex, RNAP-EcTopoI, as predicted by docking. [RNAP: Orange (RNAP β' subunit) and gold-colored structure; EcTopoI-CTD: Dark green; EcTopoI-NTD: Light green]. The red dotted circles highlight the regions of RNAP β' subunit and EcTopoI-CTD selected for simulation studies. (B) Cartoon representation of the complex (C) SPR sensogram of analyte (RNAP) binding to immobilized ligand (EcTopoI) on a CM5 sensor chip (D) Steady state response values (Req, grey shaded area in C) plotted as a function of RNAP concentration. (E) Cartoon structure of the complex, showing interfacial residues in contact after energy minimization. (F) EcTopoI-ssDNA bound structure (PDB ID: 4RUL) [Magenta: ssDNA]

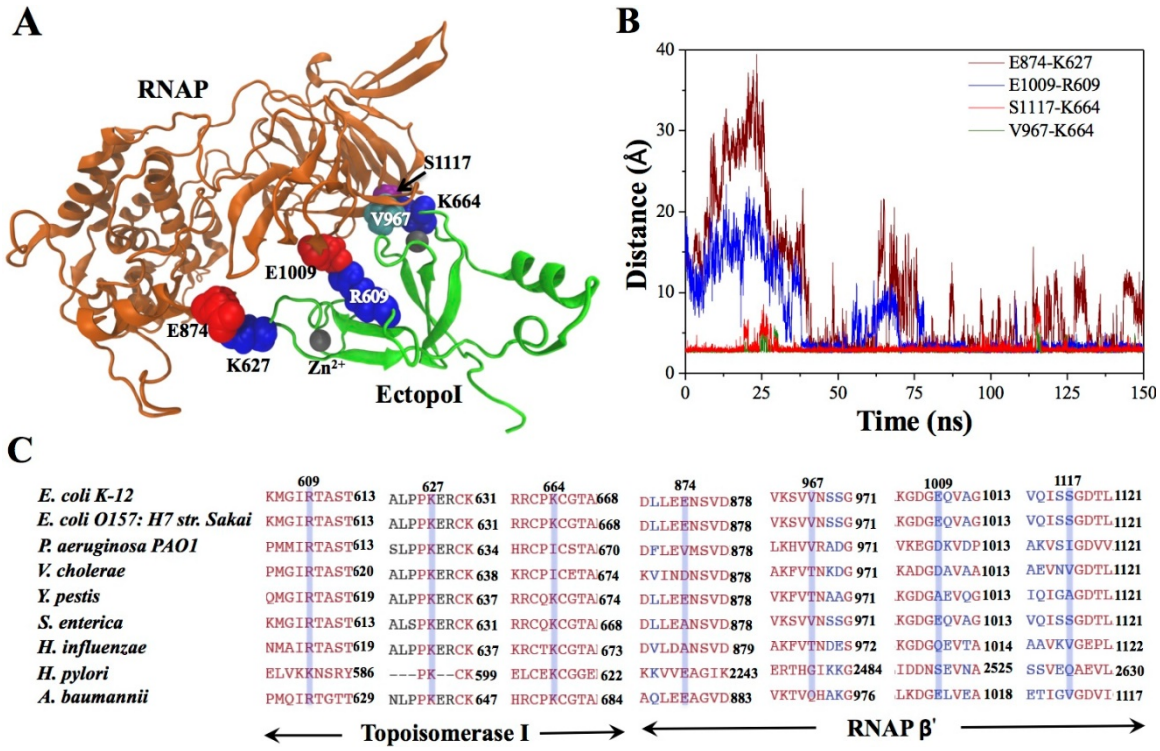


Figure 1.3. Residues involved in the EcTopoI-RNAP complex formation (Tiwari et al., 2016). (A) RNAP-EcTopoI complex after 150 ns of NVT simulations. (B) Distance between the predicted amino acid residues involved in the complex formation, over a 150 ns trajectory. (C) Sequence alignment of Topoisomerase I and RNAP β' subunit across bacteria. The residues having a potential for salt bridge and hydrogen bond formation are highlighted in blue transparent boxes.

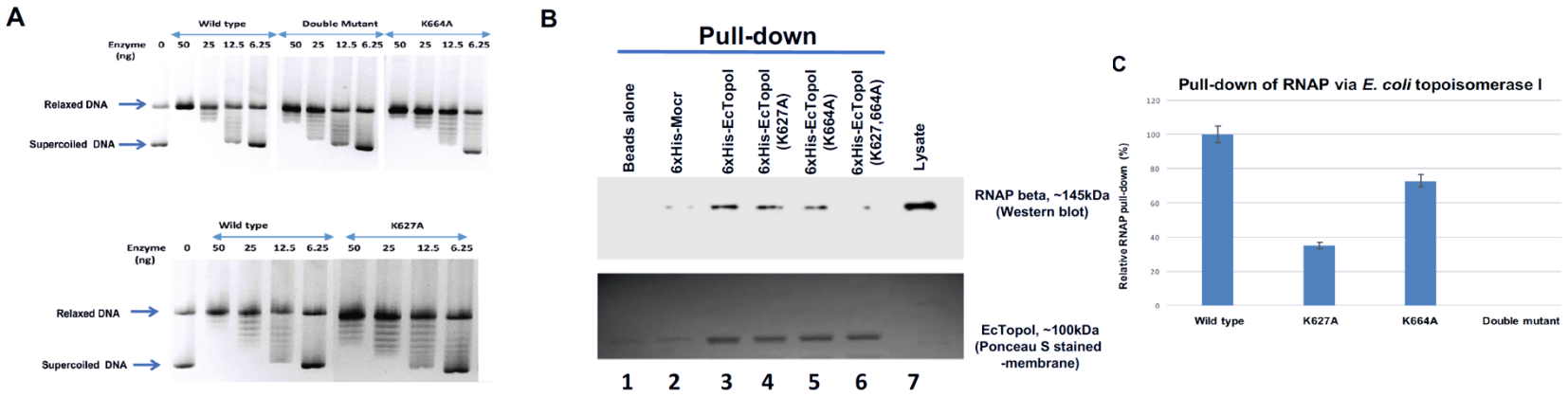


Figure 1.4. Lysine 627, 664 are essential for complex formation with RNAP. (A) Relaxation assays were carried out with Wild type, and Mutant enzymes. The reactions were set up at 37°C for 20 minutes in the presence of 5 mM $MgCl_2$ (B) Pull-down of RNAP from *E. coli* soluble lysate by recombinant wild type EcTopoI (Lane 3) or mutant EcTopoI (Lane 4, 5, 6). Negative control: Pull-down with recombinant Mocr (Lane 2). RNAP in the lysate did not by itself bind to the beads (Lane 1). (C) Graph representing average values of RNAP beta band intensities, from three independent pull-down experiments, relative to the RNAP beta band intensity in the negative control. The error bars represent standard deviations of three measurements.

CHAPTER 2

IDENTIFICATION OF PROTEIN PARTNERS OF MYCOBACTERIAL TOPOISOMERASE I

ABSTRACT

Protein-protein interactions (PPIs) drive essential cellular processes including DNA replication, transcription, translation, recombination, and repair. The activities of DNA topoisomerases are required for a normal relay of these processes. The functional association of topoisomerases with proteins involved in these processes is anticipated for maintaining the genomic stability. The physiological pathways in which topoisomerases are involved can be envisaged by identifying their protein partners. In this chapter, the protein-protein interactions of *M. smegmatis* DNA topoisomerase I, an enzyme that can relax excessive negative supercoiling, were investigated. Co-immunoprecipitation studies using antibodies against mycobacterial topoisomerase I, and pull down assays using recombinant *M. smegmatis* topoisomerase I were implemented, and the protein partners were identified by mass spectrometry. RNA polymerase was identified as a potential partner of topoisomerase I by these approaches. Putative DEAD/DEAH Box helicase (MSMEG_1930) was identified as a physical partner through Pull-down assays. Knowledge gained from this study can provide valuable insights into the physiological functions of a potential drug target, DNA topoisomerase I, in pathogenic mycobacteria.

INTRODUCTION

World health organization's global health report (2016) has declared Tuberculosis(TB) to be one of the world's deadliest communicable diseases. *Mycobacterium tuberculosis*, an aerobic rod shaped bacteria, is the causative agent of tuberculosis, although, other species of Mycobacterium (*M. bovis, M. africanum, M. canetti* etc.) can cause the disease in immune-compromised patients (Addo et al., 2007). A major roadblock, in the treatment of TB, is the development of multidrug resistant (MDR) and totally drug resistant (TDR) TB strains (Plocinski et al., 2014). Identification, and targeting of novel drug targets in the pathogenic mycobacteria could serve as a promising approach in the treatment of refractory tuberculosis.

DNA topoisomerases, topoisomerase I and gyrase, are validated drug targets in *M. tuberculosis* (Nagaraja et al., 2017). The genome-wide distribution profile of DNA topoisomerases, topoisomerase I and gyrase, relative to RNA polymerase in *M. tuberculosis* by Chromatin immunoprecipitation sequencing (ChIP-seq) revealed their localization on the active transcriptional units. The topoisomerase I and gyrase were localized behind and ahead of the RNA polymerase respectively, suggesting their role in resolving the transcription driven supercoiling (Ahmed et al., 2017). The activities of these enzymes are not just limited to the transcription process, they are believed to maintain the topology of DNA during other essential processes involving DNA transactions (Nagaraja et al., 2017; Wang, 2002). Although gyrase has been extensively targeted by clinically successful fluoroquinolones, the bacterial topoisomerase I is not yet exploited as a target for clinical antibiotics (Nagaraja et al., 2017). The development of quinolone resistance has posed a threat for the treatment of TB and other bacterial infectious diseases (Andrea

et al., 2009). Hence, topoisomerase I – the only type IA topoisomerase in mycobacteria can serve as an alternative drug target (Tse-Dinh, 2007). The presence of only one type IA topoisomerase in mycobacteria, and its essentiality for the growth and survival of the pathogen (Ahmed et al., 2014), has made this enzyme a validated TB drug target (Nagaraja et al., 2017; Ravishankar et al., 2015; Tse-Dinh, 2015). In vitro and in vivo loss in viability of *M. tuberculosis* H37Rv was reported when the intracellular topoisomerase I levels were depleted, demonstrating the essentiality of this enzyme (Ravishankar et al., 2015).

In order to fully understand the physiological function of mycobacterial topoisomerase I, elucidating the protein-protein interactions involved with this essential enzyme is critical. It would be ideal to perform studies on *M. tuberculosis,* a biosafety level-3 pathogen. Since, the department of environmental health and safety at FIU strictly prohibits working with Biosafety level 3 and 4 pathogens, a non-pathogenic and evolutionarily closely related model organism, *M. smegmatis*, was utilized for my studies. It should be noted that studies carried out on *M. tuberculosis* in chapter 3, were carried out with the whole cell lysates purchased from ATCC (NR-14822).

The topology of DNA is maintained by an important group of evolutionarily conserved enzymes called topoisomerases (Wang, 2002). The essential cellular processes including replication, transcription, recombination, and repair create topological strain on the double helix of DNA (Champoux, 2002a). Topoisomerases, acting in concert, transiently cleave and rejoin DNA to resolve the strain, and maintain the genomic stability. Topoisomerases are placed in two families, type I and type II, depending on the number of strands cleaved during each catalytic cycle. Further, in view of their structural and mechanistic differences, topoisomerases are divided in to subfamilies of IA, IB, IIA, and

IIB. In this chapter, the focus will be channeled at studying mycobacterial type IA topoisomerase. The type IA topoisomerases (excluding reverse gyrase) recognize, nick a single strand of DNA by forming a transient 5'-phospotyrosine covalent linkage, and relax the negatively supercoiled DNA via "enzyme-bridged strand passage mechanism" (Wang, 1971; Viard et al., 2007). A notable feature of *M. tuberculosis* is the presence of a single, indispensable, type IA topoisomerase. Mycobacterial topoisomerase I, thus, can serve as a potential drug target in the TB therapy. All the currently available topoisomerase targeting antibacterial drugs are inhibitors of topoisomerase II, and so far, potent and specific inhibitors against bacterial topoisomerase I remained to be discovered (Nagaraja et al., 2002). Investigating the protein partners of mycobacterial topoisomerase I, and deciphering the necessity of the interaction in the viability will open up new avenues in the treatment strategies. Targeting the interface of an essential protein-protein interaction by a novel fragment based drug discovery approach has shown promise (Scott et al., 2012, Scott et al., 2013). For instance, the interface of protein-protein interactions between a tumor suppressor BRCA2 and a recombinant enzyme RAD51 was targeted by the fragment-based approach. The disruption of BRCA2-RAD51 interaction can possibly lead to a cellular hypersensitivity to radiation and genotoxic drugs (Scott et al., 2013).

The protein partners of type IA topoisomerase may define the physiological roles of the enzyme (Viard et al., 2007). The type IA topoisomerases contain evolutionarily conserved core N-terminal domains (NTD) enclosing the active site (Wang, 1996; Berger, 1998; Champoux, 2001), and C-terminal domains (CTD) highly variable in size and sequence. The mycobacterial topoisomerase I lacks the tetracysteine zinc ribbon motifs in its CTD, unlike *E. coli* topoisomerase I. Recently, it was reported that a nucleoid associated

protein of *M. tuberculosis*, MtHU, physically and functionally interacts with the CTD of topoisomerase I. The nucleoid associated protein, HU, can stimulate the DNA relaxation ability of topoisomerase I by a direct physical interaction (Ghosh et al., 2014). According to this report, the protein-protein interaction regulates the topoisomerase I activity by enhancing the strand passage activity of topoisomerase I. The CTD of topoisomerase I was also reported to physically and functionally interact with MazF protein Rv1495. According to this report, the protein-protein interactions have a negative effect on the activities of topoisomerase I and Rv1495. The physical interaction inhibited the relaxation activity of topoisomerase I, and the mRNA cleavage activity of Rv1495 (Huang et al.,2010). Both the reports suggested a regulation of topoisomerase I activity by protein-protein interactions.

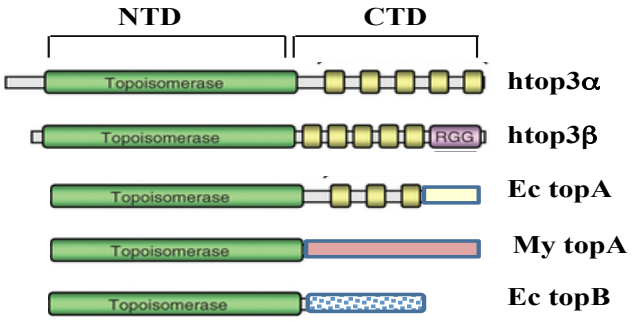


Figure 2.1. A comparison of type IA topoisomerases showing highly-conserved N-terminal domains (NTD), and variable C-terminal domains (CTD). htop3α: Human topoisomerase III alpha, htop3β: Human topoisomerase III beta, Ec topA: *E. coli* topoisomerase I, Ec topB: *E. coli* topoisomerase III, My topA: Mycobacterial topoisomerase I. Tetracysteine Zinc ribbon motif.

In the current study, novel protein partners of mycobacterial topoisomerase I were explored by a combination of pull-down and co-immunoprecipitation assays coupled to mass spectrometry. The study is based on the hypothesis that the CTD of mycobacterial topoisomerase I could be involved in protein interactions, among its other functions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cloning

M. smegmatis mc^2 155 was obtained from ATCC and cultured in LB-Tween 80 (0.1%). Overexpression of *M. smegmatis* topoisomerase I (MsmTopoI) in *E. coli* BL21 STAR DE3 (Invitrogen) for protein purification was achieved from a vector, pET His6 Mocr TEV LIC cloning vector (2O-T, Addgene Plasmid# 29710), Gibson cloned with MsmtopA gene. *M. smegmatis* RNAP beta (RpoB), RNAP beta' (RpoC), Putative DEAD/DEAH Box helicase (MSMEG_1930), and *M. tuberculosis* H37Rv Putative ATP dependent RNA helicase (RhlE) genes were Gibson cloned separately into the SspI site of pLIC-HK, and transformed into *E. coli* SoluBL21 (Genlantis) cells for protein expression and purification. The PCR primers used in the cloning are listed in the Table 2. T1.

Protein expression and purification

The recombinant histidine tagged proteins – full length *M. smegmatis* DNA topoisomerase I (MsmTopoI) and fragment containing the C-terminal domains of Topoisomerase I (MsmTopoI-CTD) were purified initially for the pull-down assays with the *M. smegmatis* soluble lysate. Later, the potential partners of topoisomerase I, identified by coupling mass spectrometry to the pull-down assays, were purified for verifying a direct physical interaction. Basing on the mass spectrometry data, recombinant histidine tagged proteins of *M. smegmatis* RNAP beta subunit, RNAP beta' subunit, Putative DEAD/DEAH Box helicase were purified for further studies. A homologue of putative DEAD/DEAH Box helicase in *M. tuberculosis* H37Rv, ATP-dependent RNA helicase, was also expressed and purified with a recombinant Histidine tag for further studies. Overexpression of these

recombinant proteins in the *E. coli* hosts was induced with 1 mM IPTG. Following the overexpression and cell lysis, recombinant proteins were purified to near homogeneity by affinity chromatography using Ni-Sepharose 6 Fast flow beads (GE Healthcare Life Sciences) followed by size-exclusion chromatography. The details of the purification are elaborated in chapter 3.

Relaxation assay

The *in vitro* relaxation activity of the purified topoisomerase I was tested. A supercoiled pBAD/Thio plasmid DNA substrate (160 ng), purified by cesium chloride gradient centrifugation method, was incubated with different amounts of the enzyme in the presence of 5mM $MgCl_2$ (Sandhaus et al., 2016). The reaction mixture was setup at 37°C for 30 minutes. The reaction was terminated by the addition of stop buffer (50 mM EDTA, 50% glycerol, 0.5% v/v bromophenol blue), and the reaction products were electrophoresed on a 1% Agarose gel and visualized over UV light following ethidium bromide staining. The effect of a putative helicase on the relaxation activity of *M. tuberculosis* topoisomerase I was also verified (Figure 2.7, B).

Table 2. T1. PCR primers for Gibson cloning

Primer	Gene	Sequence (5'-3')
MsmTopoI_2OT_FP	MsmtopA	GGGATCGAGGAAAACCTGTACTTCCAAATG GCTGGCGGCGACCG
MsmTopoI_2OT_RP	MsmtopA	GCGGATCCGTTATCCACTTCCAATATTGTTC GGCGGAAACCTAGGCCTTCTT
RNA Polymerase beta_LIC_FP	rpoB	CTGTACTTCCAATCCAATGTGCTGGAAGG ATGCA
RNA Polymerase beta_LIC_RP	rpoB	ATCCGTTATCCACTTCCAATCTACGCGAGA TCCTCGAC
RNA Polymerase beta'_LIC_FP	rpoC	CTGTACTTCCAATCCAATGTGCTAGACGT CAACTTC
RNA Polymerase beta'_LIC_RP	rpoC	CGTTATCCACTTCCAATTTAGCGGTAATCC GAGTAG
Putative DEAD/DEAH_LIC_FP	MSMEG_1930	CTGTACTTCCAATCCAATATGACGCAACTCAATCACT CGTTTGC
Putative DEAD/DEAH_LIC_RP	MSMEG_1930	CGTTATCCACTTCCAATTCAGCCTGCGGTGGCTGC
Putative RhlE_LIC_FP	RhlE	CTGTACTTCCAATCCAATATGACCGCAGTGAAACAC ACAACTG
Putative RhlE_LIC_RP	RhlE	CGTTATCCACTTCCAATTCAGTTAGCCCGCGCGGC

Purification of *M. tuberculosis* topoisomerase IgG from the Rabbit Serum

The antibodies from the serum of a Rabbit were purified with Promega Protein A magnetic beads (G8781). In the Co-Immunoprecipitation assays, purified antibodies against topoisomerase I were used for precipitating the topoisomerase I complex from the lysate. A rabbit was injected with *M. tuberculosis* topoisomerase I (MtbTopoI), and the serum was collected for verification of the MtbTopoI IgG production. As a control, serum was also collected prior to the MtbTopoI injection. Western blots were performed to verify the antibodies in the serum. The serum, control and antibody containing, was used for isolation of IgG that will be later used in the Co-IP. The antibody purification protocol is detailed in chapter 3. The purified MtbTopoI IgG can cross-react with *M. smegmatis* topoisomerase I, MsmTopoI (Figure 3.3).

Co-Immunoprecipitation (Co-IP)/ Tandem Mass spectrometry

The complex of *M. smegmatis* topoisomerase I (MsmTopoI) and its potential protein partners were isolated from the *M. smegmatis* soluble cell lysate by Co-IP (Free *et al.*, 2009). *M. smegmatis* mc^2 155 was grown to stationary phase in LB-Tween 80 (0.1%). The pelleted cells were resupsended in lysis/wash buffer (50 mM NaCl, 50 mM NaH_2PO_4, pH 8.0, 0.1% NP-40), and then subjected to 5 pulses of sonication (10 seconds/pulse at medium intensity). The lysate was spun at 16000xg for an hour, and the supernatant soluble lysate was used as prey in the Co-IP assays. The soluble lysate was pre-incubated (pre-cleared) with protein A/G plus Agarose beads to remove proteins that bind non-specifically to the beads. The pre-cleared lysate (total protein=500 μg) was first mixed with IgG (10 μg) purified from serum of a rabbit inoculated with MtbTopoI or control pre-immune

serum. The rabbit MtbTopoI polyclonal antibodies can cross react with MsmTopoI (Figure 3.3). The Protein A/G plus agarose beads (Santa Cruz), previously equilibrated in wash buffer, were then added to the lysate-antibody reaction and left overnight at 4°C. On the following day, the reaction mixture was spun at 700xg, and the supernatant was discarded. The bead pellet, with the bound proteins, was washed thrice in the wash buffer. The elution of the proteins bound to the beads was carried out by suspending the bead pellet in 4X SDS sample buffer (240 mM Tris-HCl, 8% SDS, 40% glycerol, 0.04% bromophenol blue), and heating at 95°C for 2 minutes. The eluted proteins (MsmTopoI, and its potential protein partners) were stained with coomassie blue following SDS PAGE. The protein bands of interest were excised for characterization by tryptic digest and mass spectrometry (nano LC/MS/MS) at the Proteomics and Mass Spectrometry Facility of University of Massachusetts Medical School. The MS/MS results were searched using Mascot (Matrix Science, London, UK; version 2.5.0) against the UniProt_MSmegmatis_071714 database for trypsin digestion products.

Pull-down assay/ Tandem Mass spectrometry

For the pull-down assay (Brymora et al., 2004), a high concentration of the target protein (MsmTopoI) with a fusion tag (N-terminal 6xHistidine) was mixed with *M. smegmatis* mc^2 155 soluble cell extract for 2 hours at 4°C, and then immobilized on a HisPur Cobalt Agarose resin (Thermofisher). The resin with bound target proteins was washed thrice in a pull-down wash buffer (10 mM HEPES, pH 7.5, 10 mM Imidazole, 0.005% Tween-20) to remove the weakly retained proteins. The proteins that remained bound to the resin were eluted with pull-down elution buffer (10mM HEPES, pH 7.5, 350

mM Imidazole), separated by SDS PAGE and stained with coomassie blue. The protein bands of interest were excised for identification by tryptic digest and nano LC/MS/MS.

Physical interactions of Topoisomerase I with putative helicase, and RNA Polymerase

RNA polymerase, and putative DEAD/DEAH box helicase were identified in the protein-protein interaction network of *M. smegmatis* topoisomerase I. However, the proteins may or may not be involved in direct physical interactions with topoisomerase I. The physical interactions, if any, between two proteins can be assayed by the incubation of proteins (as bait and prey) in the Pull-down (Banda et al., 2016). The fusion tag [6xHis-Mocr] of purified prey protein was cleaved prior to their use in the assay (Phan et al., 2002). Briefly, the bait (His tagged putative helicase or RNA polymerase subunit) and the prey (MsmTopoI) were incubated together for 2 hours at 4°C. The bait-prey complex was trapped with HisPur cobalt resin, and washes were carried out with the pull-down wash buffer. The bait-prey complex was then eluted from the resin with the pull-down elution buffer. The eluate was resolved on a SDS-PAGE, and immunoblotted with antibodies against mycobacterial topoisomerase I.

Helicase Assays

The putative helicase identified as a protein partner of topoisomerase I has not been characterized before. The protein is believed to be a putative helicase based on the sequence homology with DEAD box helicase family of proteins. The purified putative helicase was tested for its DNA or RNA helicase activity. Synthetic oligonucleotides were ordered from Sigma for constructing the potential substrates of the putative helicase. A 5' ^{32}P labeled

DNA or RNA strand was created by incubating a synthetic oligonucleotide with T4 polynucleotide kinase, and [γ-^{32}P]. The labeled oligonucleotide was heated at 95°C to inactivate the kinase. 3-fold excess of a complementary DNA or RNA was added, and allowed to anneal with labeled strand. A 5' over hang, 3' over hang and blunt ended substrates were constructed for the verification of helicase activity. The helicase reaction mix (10 µl) contains 20 mM Tris-HCl; pH: 8.0, 5 mM $MgCl_2$, 0.2 pmole of the radiolabeled substrate, and the putative helicase. The reaction was pre-incubated at room temperature for 10 minutes, before initiating the unwinding reaction with 3 mM ATP and a 10-fold excess of the unlabeled oligonucleotide (trap strand) identical to the labeled strand. The trap strand prevents the reannealing of the unwound strand to the labeled strand. The reaction was set up at 37°C for 45 minutes. The reaction was quenched with a buffer containing 1% SDS, 20 mM EDTA, 50% Glycerol, and 0.3% Bromophenol blue. The reaction products were electrophoresed on a 20% native polyacrylamide gel, and visualized by autoradiography (Uson et al., 2015).

RESULTS

Identification of RNA Polymerase, and putative helicases as protein-protein interaction partners of MsmTopoI

Protein interaction network of the target protein (MsmTopoI) was analyzed by two different approaches: Co-IP, and Pull-down assays coupled to Mass spectrometry. The *M. smegmatis* topoisomerase I complex was isolated from *M. smegmatis* soluble lysate by either of the approaches, electrophoresed on a SDS polyacrylamide gel, and coomassie stained (Figure 2.2, B; Figure 2.3, B). The efficiency of the antibodies raised against

MtbTopoI to immunoprecipate MsmTopoI from the cell lysate was confirmed by western blot analysis of the Co-IP eluates (Figure 2.2, C). LC MS/MS analysis of the protein bands, a-d (Lane 5 of Figure 2. 2, B), identified *M. smegmatis* RNAP beta and beta' subunits in protein band "a" and a putative helicase (MSMEG_2174) in the band "b". Proteins involved in the metabolism of DNA, sugars, and lipids were also identified as protein partners by Co-IP (Table 2. T2). Alternatively, the potential protein partners of *M. smegmatis* topoisomerase I were also identified by analyzing the protein complexes pulled down with His-tagged MsmTopoI and MsmTopoI-CTD. Coomassie-stained proteins, e-f (lanes 7 and 8 of Figure 2.3, B), pulled down by either His-tagged MsmTopoI or MsmTopoI-CTD, but not control His-Mocr (lane 6, Figure 2.3, B) were analyzed by LC MS/MS. *M. smegmatis* RNA polymerase beta and beta' subunits were again identified in protein band "e". Interestingly, Acetyl-/propionyl-coenzyme A carboxylase, a protein involved in the lipid metabolism was identified as potential partner of *M. smegmatis* topoisomerase I by both the Co-IP, and pull-down approaches. Helicase partners (Putative DEAD/DEAH Box helicase, ATP-dependent helicase HrpA, ATP dependent RNA helicase DeaD) were also identified by pull-down approach (Figure 2.3, B). However, it is essential to note that these helicase partners were not identified by the Co-IP approach.

Physical interactions between RNA polymerase beta' and Topoisomerase I

There is a high degree of confidence, based on Co-IP and Pull-down (Figure 2.2, 2.3), for the association of topoisomerase I with the RNA polymerase. However, the evidence for a physical interaction was not obtained from these experiments. The direct physical interactions were analyzed by purified recombinant proteins. A Physical

interaction between *M. smegmatis* RNA polymerase beta' subunit and *M. smegmatis* topoisomerase I was verified by both co-immunoprecipitation (Figure 3.6, A) and Pull-down experiments (Figure 3.6, B). This protein-protein interaction is specific. No interaction with MsmTopoI could be observed for *M. smegmatis* RNA polymerase beta subunit (Figure 3.6, A). The physical interactions of topoisomerase I, and RNA polymerase beta' subunit has shown species specificity. *E. coli* topoisomerase I was has not shown interactions with *M. smegmatis* RNA polymerase beta' subunit (Figure 3.6, C).

Physical interactions between DEAD/DEAH box helicase and Topoisomerase I

Putative DEAD/DEAH Box helicase was identified in the protein interaction network of topoisomerase I by pull-down assays (Figure 2.3, band f). The putative helicase has a homologue, RhlE, in *M. tuberculosis* H37Rv. Topoisomerase I from *M. tuberculosis* H37Rv and *M. smegmatis* have a high degree of homology. Homology among these proteins can indicate a potential conservation of protein-protein interactions. Here, the physical interaction between the putative helicase and topoisomerase I of *M. smegmatis*, *M. tuberculosis* H37Rv was verified by pull-down assay. The assays have provided evidence for a physical interaction between putative helicase and topoisomerase I in *M. smegmatis, M. tuberculosis* (Figure 2.5)

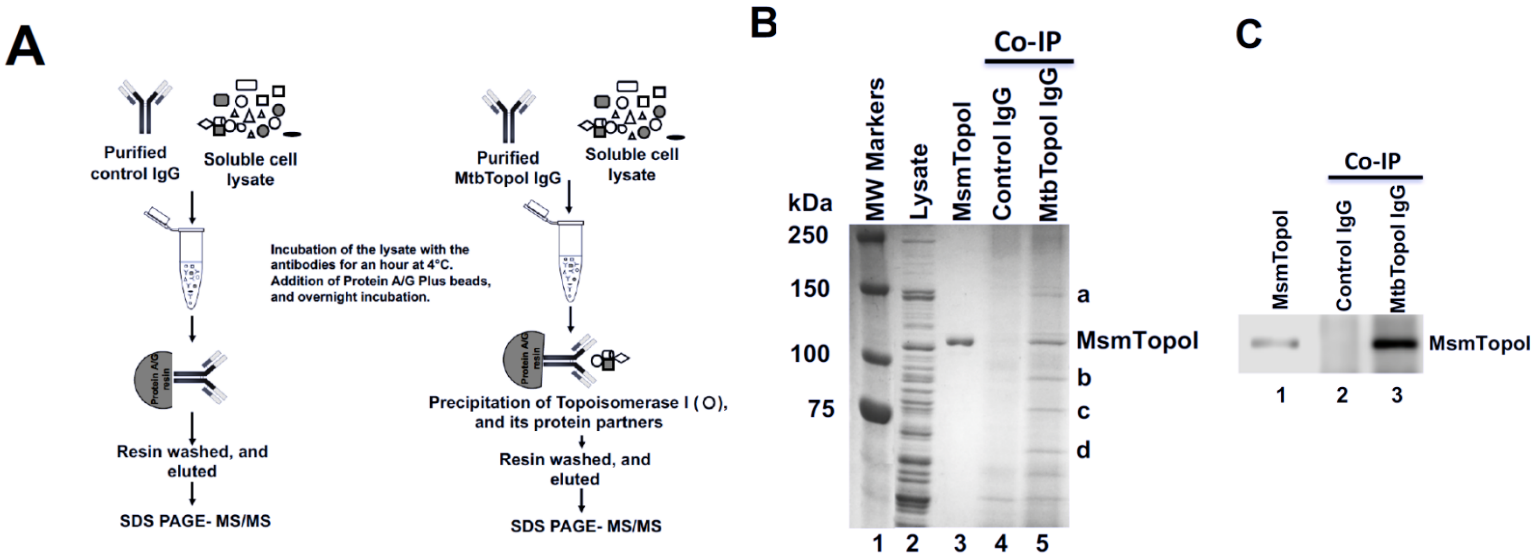


Figure 2.2. Co-immunoprecipitation of topoisomerase I and interacting proteins from *M. smegmatis* lysate. (A) Schematic of the Co-IP. (B) Proteins were stained with Coomassie blue following SDS-PAGE. Lane 1: MW standards. Lane 2: 15 µg of total *M. smegmatis* proteins in soluble lysate. Lane 3: purified recombinant MsmTopoI. Proteins were immunoprecipitated from *M. smegmatis* lysate (500 µg total proteins) by pre-immune rabbit antibodies (Lane 4) or antibodies raised against MtbTopoI (Lane 5). Bands a-d were selected for LC MS/MS analysis. (C) A fraction of the eluates from Co-IP reactions were electrophoresed on a SDS-PAGE, and immunoblotted with TopoI antibodies. Lane 1: 25 ng of Purified MsmTopoI. The efficiency of the Co-IP assay was verified by analyzing a small fraction of eluates from the reaction of *M. smegmatis* lysate with pre-immune rabbit antibodies (Lane 2), or antibodies raised against MtbTopoI (Lane 3).

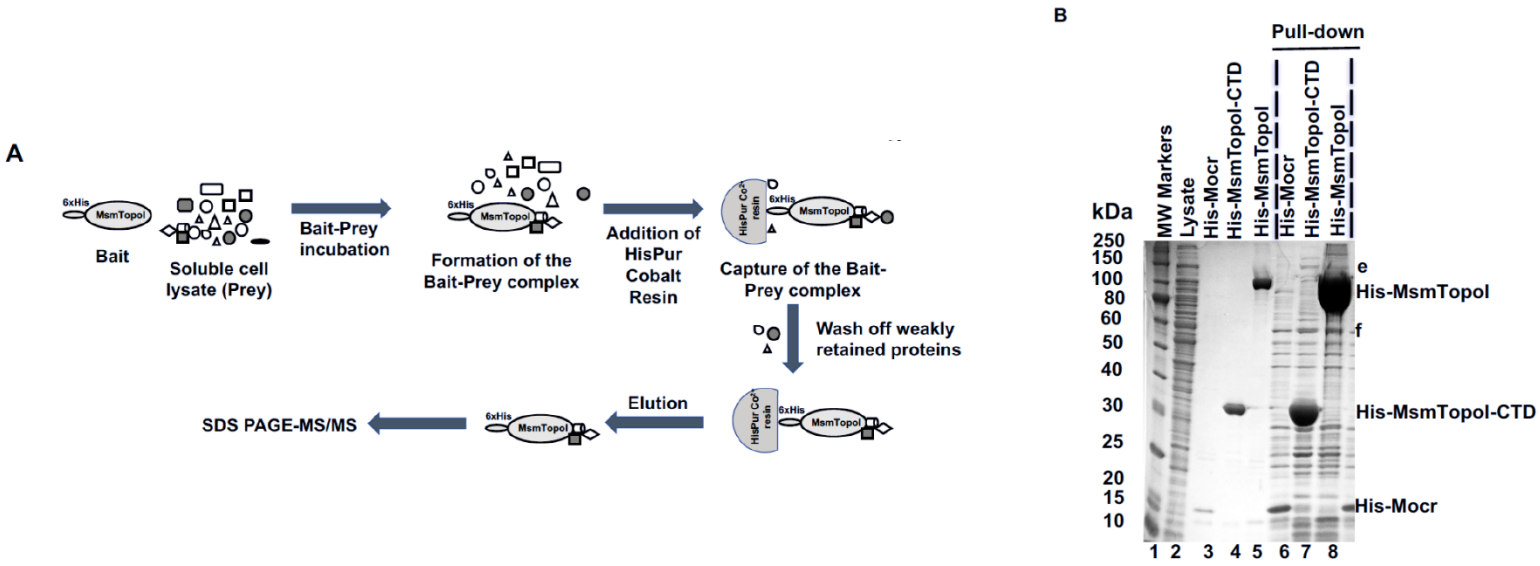


Figure 2.3. Pull-down assay of MsmTopoI-RNAP interaction with HisPur Cobalt agarose resin. (A) General scheme of the Pull-down assay. (B) Proteins pulled down from *M. smemgatis* soluble lysate by recombinant N-terminal His-tagged MsmTopoI (Lane 8) or MsmTopoI-CTD (Lane 7). His-Mocr, a recombinant viral protein, was used as a bait in the control reaction (Lane 6). Lane 1: MW standards; Lane 2: purified recombinant His-Mocr; Lane 3: purified recombinant His-MsmTopoI-CTD; Lane 4: purified recombinant His-MsmTopoI.

Table 2. T2. Potential protein partners of topoisomerase I identified by Co-IP/MS

Band (Figure 1)	Proteins identified by MS/MS
a	1. RNA Polymerase beta (rpoB), 2. RNA Polymerase beta' (rpoC) 3. Alpha-(1->3)-arabinofuranosyltransferase (aftD)
b	1. Putative helicase (MSMEG_2174) 2. Glycoside hydrolase, family 3-like protein (MSMEG_5144) 3. Phosphoribosyl formylglycinamidine synthase (purL) 4. DNA translocase FtsK (MSMEG_2690) 5. Drug exporters of the RND superfamily-like protein (mmpL3)
c	1. Uncharacterized protein (MSMEI_5149) 2. Proteasome-associated ATPase (mpa) 3. Transglycosylase (MSMEG_6201)
d	1. Serine/threonine-protein kinase (MSMEG_5513) 2. Acetyl-/propionyl-coenzyme A carboxylase alpha chain (MSMEG_0334) 3. Acetyl-/propionyl-coenzyme A carboxylase alpha chain (accA2) 4. Succinate dehydrogenase flavoprotein (MSMEG_0418)

Table 2. T3. Potential protein partners of topoisomerase I identified by Pull-down/MS

Band (Figure 2)	Proteins identified by MS/MS
e	1. RNA Polymerase beta (rpoB) 2. RNA Polymerase beta' (rpoC) 3. ATP-dependent helicase HrpA (MSMEI_6410)
f	1. DEAD/DEAH box Helicase (MSMEG_1930) 2. Dihydroxyacetone kinase (MSMEG_3271) 3. Pyruvate dehydrogenase (MSMEG_2280) 4. Transcriptional regulator (MSMEI_6320) 5. Propionyl coA carboxylase beta chain (accD5) 6. Oxoalyl coA decarboxylase (oxcA) 7. ABC Transporter ATP-binding protein (MSMEG_3140) 8. Phosphoenolpyruvate-protein phosphotransferase (ptsI) 9. Phosphoglucomutase/Phosphomannomutase (pmmB) 10. RelA/Spot domain protein (MSMEG_5849) 11. ATP-dependent RNA helicase DeaD (deaD) 12. Acetyl-/propionyl-coenzyme A carboxylase alpha chain (MSMEG_0334) 13. Fatty-acid-CoA ligase FadD6 (FadD6)

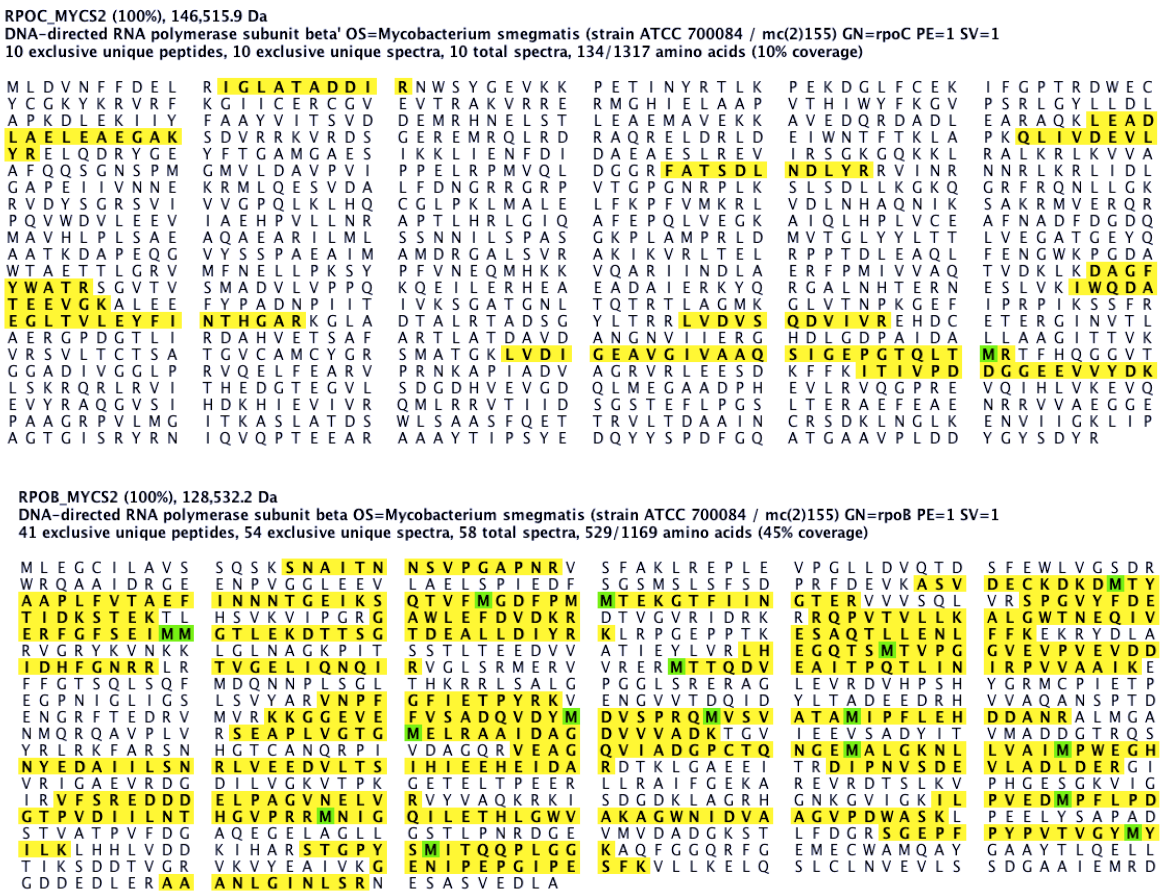


Figure 2.4. Unique peptides of RNAP subunits (highlighted) identified by mass spectrometry of the eluates from the Co-IP assay.

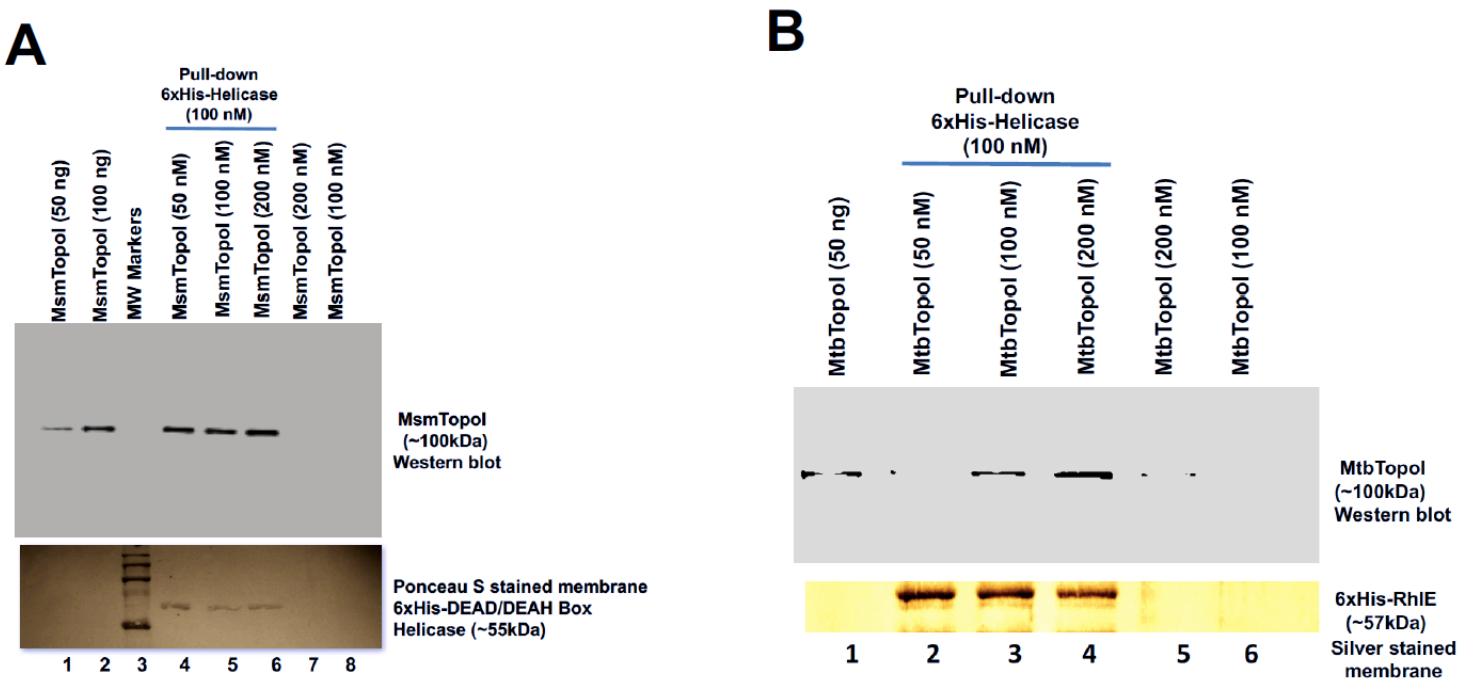


Figure 2.5. Physical interaction of putative DEAD/DEAH Box helicase with topoisomerase I. (A) Pull-down: *M. smegmatis* topoisomerase I, MsmTopoI, can directly interact with putative DEAD/DEAH Box helicase (Lane 4,5,6). MsmTopoI did not by itself bind to the resin (Negative control, lane 7&8). (B) Pull-down: *M. tuberculosis* topoisomerase I, MtbTopoI, can directly interact with putative helicase, RhlE (Lane 2,3,4). Lanes 5&6: negative control.

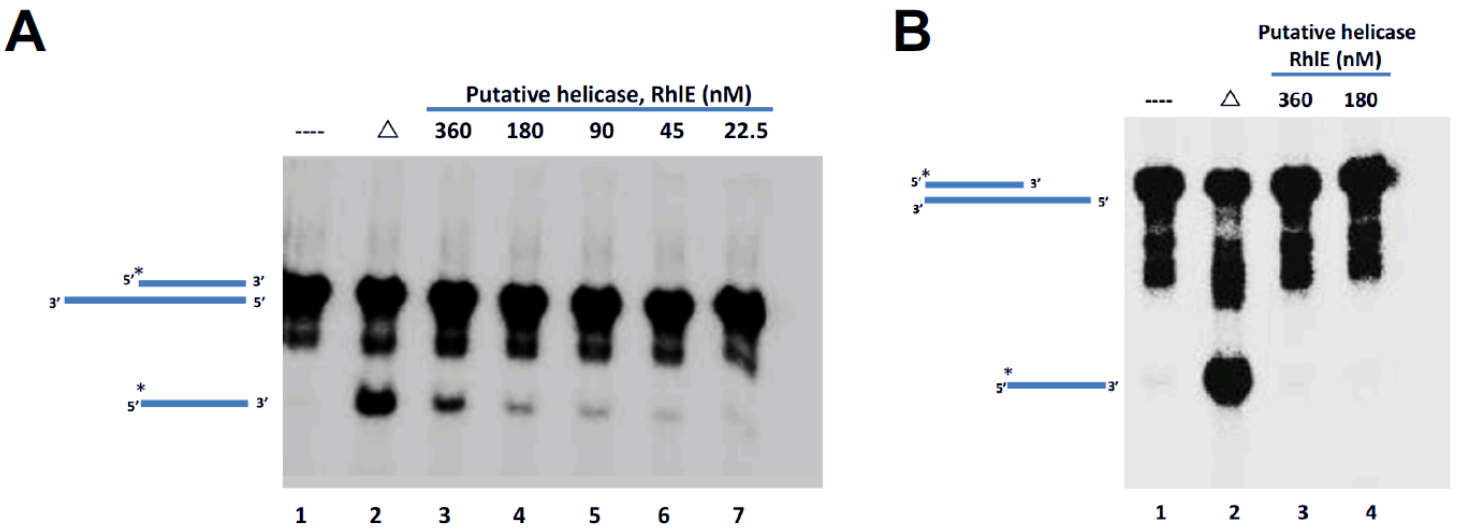


Figure 2.6. Putative helicase, RhlE, has a 3'-5' DNA helicase activity. Products from the helicase assays were electrophoresed on a 20% polyacrylamide gel. (A) Helicase activity was verified with a DNA substrate having a 3' overhang. Lane 1: The product of incubating the substrate with the reaction mix (No enzyme). Lane 2: Product from heating the substrate at 95°C for 15 minutes. Lane 3-7: Products of substrate incubation with the indicated amounts of putative helicase. (B) Verification of the helicase activity on a substrate with 5' overhang. Lane 1: Product of substrate incubated with the reaction mix (No enzyme). Lane 2: The product of the substrate heated at 95°C for 15 minutes. Lanes 3, 4: The products from the treatment of substrate with the indicated amounts of putative helicase.

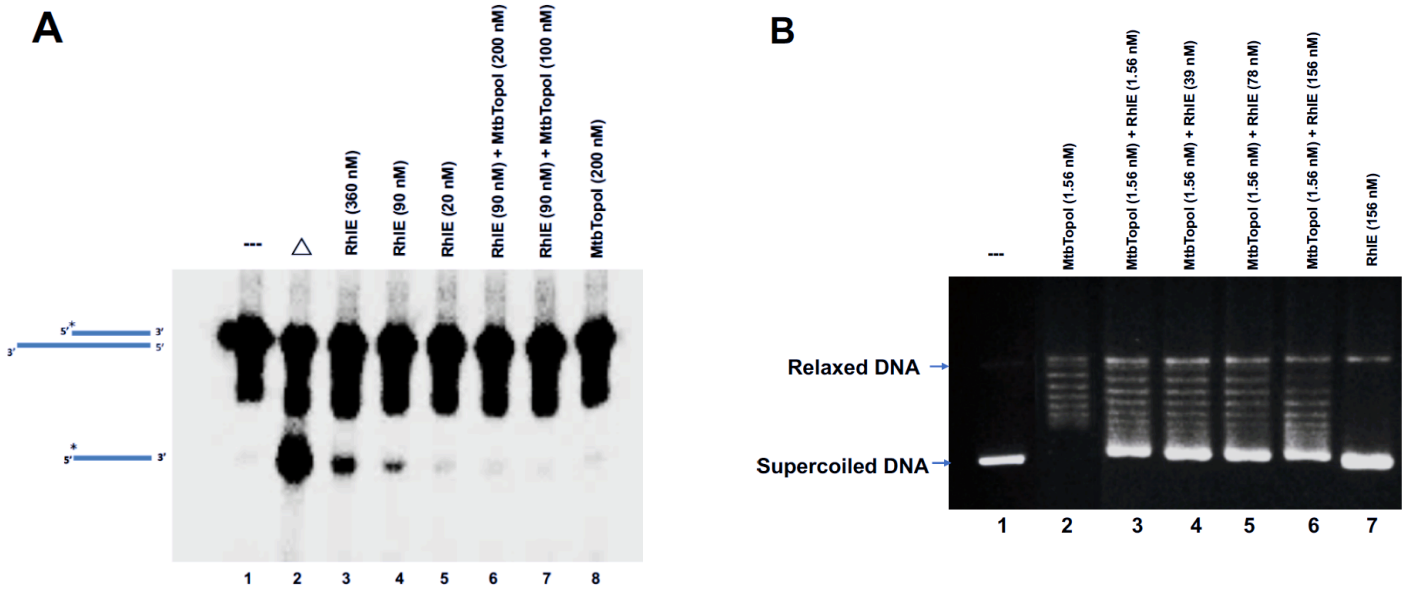


Figure 2.7. Putative helicase –RhlE and *M. tuberculosis* topoisomerase I (MtbTopoI) have a mutual inhibitory effect on their activities. (A) Helicase assays were carried out in the presence and absence of MtbTopoI. A 3'-overhang DNA substrate was used. Products from the assays were electrophoresed on a 20% native polyacrylamide gel. Lane 1: Product of incubating DNA substrate with the reaction mix (No putative helicase); Lane: Product of heating the DNA substrate at 95°C for 15 minutes; Lanes 3-5: Products of incubating the substrate with the indicated amounts of putative helicase; Lane 6,7: Product of incubating the substrate with the indicated amounts of putative helicase and topoisomerase I; Lane 8: Product of incubating the substrate with the indicated amount of topoisomerase I (B) Relaxation activity of MtbTopoI was verified in the presence and absence of the putative helicase. Lane 1: Supercoiled DNA; Lane 2: Product of incubating supercoiled DNA with the indicated amount of MtbTopoI; Lane 2-6: Product of incubating supercoiled DNA with the indicated amounts of MtbTopoI and RhlE; Lane 7: Product of incubating supercoiled DNA with the indicated amount of RhlE.

Putative helicase, RhlE, has a 3'-5' DNA helicase activity

The DEAD/DEAH Box helicase of *M. smegmatis*, and RhlE of *M. tuberculosis* are putative helicases. Their helicase activity has not been verified before, and they have been considered as putative helicases because of the helicase motifs in their amino acid sequence. Helicase assays, in the presence of ATP and Mg (II), were carried out to verify the helicase activities. While RhlE has shown a 3'- 5' DNA helicase activity (Figure 2.6), its homologue (DEAD/DEAH Box helicase of *M. smegmatis*) did not show this activity under similar experimental conditions. RhlE also did not show any helicase activity on a DNA substrate with blunt ends or a 5' over hang. Assays were performed to verify the influence of topoisomerase I on the helicase activity, and vice versa. The results suggested a mutual inhibition of their activities (Figure 2.7). However, it could most likely be possible that the mutual inhibitory effect of the enzymes is the result of a competition for binding the DNA substrate.

DISCUSSION

The protein-protein interactions of *M. smegmatis* topoisomerase I were studied by two approaches; Co-IP, and pull-down assays coupled to mass spectrometry (Brymora et al., 2004; Free et al., 2009). We anticipated identification of similar protein partners with either of these approaches. However, the mass spectrometry data revealed almost entirely different partners for topoisomerase I, depending on the approach (Tables 2. T2, 2. T3). The dynamic nature of protein-protein interactions, and the vigorous washing steps involved with these assays could have played a part here. Interestingly, RNA polymerase beta and beta' subunits were identified as potential partners by both the approaches, giving

us a higher confidence in the interaction of RNA polymerase with topoisomerase I. The physical interaction between RNAP and topoisomerase I was reported in *E. coli* (Cheng et al., 2003), and evidence provided here verified the conservation of this interaction in mycobacteria. The pull-down approach (Lane 7 of Figure 2.3, B: band e) coupled to mass spectrometry has suggested that the C-terminal domain of MsmTopoI can interact with RNA polymerase by itself. *E. coli* topoisomerase I requires tetracysteine zinc ribbon motifs for interaction with RNAP beta' subunit. Since mycobacterial topoisomerase I lacks the tetracysteine motifs, a novel mechanism of interaction was predicted. The mechanism, and physiological relevance of the RNAP-topoisomerase I interaction is reported in greater detail in chapter 3.

Apart from RNAP, proteins involved in the metabolism of DNA, sugars, and lipids were among the identified partners. The association of topoisomerases with helicases could have a physiological significance, as the interaction could assist in the progression of replication fork, chromosome segregation, recombination, repair and genomic stability (Duguet, 1997). Evolutionarily conserved RecQ helicases have a functional association with type IA topoisomerases (Mankouri and Hickson, 2007; Vindigni and Hickson, 2009). In yeast, RecQ helicases and topoisomerase III have a physical and functional interaction (Gangloff et al., 1994, Ellis et al., 1995). In humans, topoisomerase IIIα is recruited by a RecQ helicase, BLM, to resolve the non-canonical DNA structures (Double holliday junctions, D-loops, G-quadruplexes) in cells (Wu and Hickson, 2003; Hu et al., 2001; Wu et al., 2000; Johnson et al., 2000). The human topoisomerase IIIα – BLM complex also contained a third protein, RMI1 (Hoatlin et al., 2005). The association of topoisomerase IIIα-BLM-RMI1 is essential for the maintenance of genomic integrity (Chang et al., 2005;

Mullen et al., 2005). Like topoisomerase IIIα, the physical and functional association of topoisomerase IIIβ have also been identified. The proposed role of topoisomerase IIIβ in resolving the R-loops formed during the transcription process, as a partner in a complex containing a tudor-domain containing protein (TDRD3) further implicates the role of topoisomerase interactions in genomic maintenance (Yang et al., 2014).

With the Co-IP and pull- down assays, identification of novel helicases and recently identified partners of *M. smegmatis* topoisomerase I: 3-Methyladenine DNA glycosylase (Yang et al., 2012), and D-ribokinase (Yang et al., 2011) was anticipated as possibilities. Since the Co-IP assays were performed with antibodies that were not cross-linked to beads, the denatured eluates of the assay were expected to have the light and heavy chains of the IgG along with the potential protein partners. The IgG bands in the coomassie stained gel have hindered identification of protein partners, 3-Methyladenine DNA glycosylase and D-ribokinase, which are in the same molecular weight range. Similarly, pull-down assay (Figure 2.3) was problematic in the analysis of protein partners of lower molecular weight due to very high non-specific binding of a number of proteins to the beads, as evident from the control experiment. Novel helicases (Putative DEAD/DEAH Box helicase, ATP-dependent helicase HrpA, ATP-dependent RNA helicase deaD, putative helicase MSMEG_2174) were identified as partners of *M. smegmatis* topoisomerase I. In the future, it will be interesting to study the physiological relevance of helicase-topoisomerase I interactions in the survival of mycobacteria.

CHAPTER 3

EVOLUTION OF A NOVEL MECHANISM FOR MYCOBACTERIAL RNA POLYMERASE AND TOPOISOMERASE I INTERACTION

ABSTRACT

Protein-protein interactions are the key elements of cellular processes including DNA replication, transcription, recombination and repair. In this study, we report a novel mechanism of interaction between topoisomerase I, and DNA-dependent RNA polymerase in *Mycobacterium tuberculosis* and *Mycobacteria smegmatis*. Bacterial DNA topoisomerase I is responsible for preventing the hyper-negative supercoiling of genomic DNA. The association of topoisomerase I with RNA polymerase during transcription elongation could efficiently relieve transcription driven negative supercoiling. Our results demonstrate a direct physical interaction between the C-terminal domains of topoisomerase I (TopoI-CTD) and the beta' subunit of RNA polymerase of *M. tuberculosis* and *M. smegmatis* in the absence of DNA. The TopoI-CTD in mycobacteria are evolutionarily unrelated in amino acid sequence and 3-dimensional structure to the TopoI-CTD found in the majority of bacterial species outside Actinobacteria, including *Escherichia coli*. The functional interaction between topoisomerase I and RNA polymerase has evolved independently in mycobacteria and *E. coli,* with distinctively different structural elements of TopoI-CTD being utilized for protein-protein interaction. Instead of zinc ribbon motifs seen for *E. coli* topoisomerase I, the 27 amino acid tail rich in basic residues at the C-terminal end of *M. smegmatis* topoisomerase I is responsible for the interaction with RNA polymerase. Overexpression of the TopoI-CTD as a recombinant protein in *M. smegmatis*

competed with TopoI for protein-protein interaction with RNA polymerase. The TopoI-CTD overexpression resulted in a decreased survival following treatment with antibiotics and hydrogen peroxide, supporting the importance of the protein-protein interaction between topoisomerase I and RNA polymerase during stress response of mycobacteria.

INTRODUCTION

Topoisomerases are essential enzymes responsible for control of DNA topology and facilitating vital cellular processes that include replication, transcription, recombination and DNA repair (Vos et al., 2011; Chen et al., 2013). The active site for DNA cleavage and rejoining by type IA topoisomerases is evolutionarily conserved at the N-terminal domains of bacterial topoisomerase I and topoisomerase III (Baker et al., 2009). Bacterial topoisomerase I is responsible for relieving the transcription driven negative supercoiling, generated behind the RNA polymerase complex during transcription elongation (Liu et al., 1987; Wu et al., 1988; Masse et al., 1999a). The absence of topoisomerase I activity in *Eshcherichia coli* has been shown to result in increased R-loop formation via the stable association of the nascent transcript with the unwound DNA (Drolet et al., 1995; Masse et al., 1999b). This could potentially block transcription elongation (Hraiky et al., 2000). A direct association between RNA polymerase and topoisomerase I would facilitate gene expression at highly transcribed loci, including genes induced for survival in stress response.

It was previously reported that *E. coli* RNA polymerase beta' subunit interacts directly with the zinc ribbon and zinc ribbon-like C-terminal domains (CTD) of topoisomerase I (Cheng et al., 2003; Tiwari et al., 2016). In *E. coli* and most bacterial

species, the CTD of topoisomerase I have multiple zinc ribbon domains, each with a Zn(II) ion coordinated by four cysteines (Tse-Dinh et al., 1988; Suerbaum et al., 1998; Grishin, 2000). In contrast, the topoisomerase I proteins in Acintobacteria, including mycobacteria, evolved with TopoI-CTD that do not have zinc ribbon or zinc ribbon-like domains (Ahmed et al., 2013). Structural determination by X-ray crystallography showed that the *Mycobacterium tuberculosis* topoisomerase I CTD are formed by repeats of a novel protein fold of a four-stranded antiparallel β-sheet stabilized by a crossing-over α-helix (Tan et al., 2016).

We speculated that the CTD of Mycobacterial topoisomerase I could be involved in protein-protein interactions, among its other functions. The protein-protein interactions of topoisomerase I from *M. smegmatis* were studied by co-immunoprecipitation (Co-IP), and pull-down assays coupled to tandem mass spectrometry (Brymora et al., 2004; Free et al., 2009). The assays identified an interaction between DNA-dependent RNA polymerase (RNAP), and topoisomerase I in *M. smegmatis*. This TopoI-RNAP interaction also employed the CTD of topoisomerase I even though the mycobacterial topoisomerase I CTD do not have zinc ribbon. The conservation of this interaction in *M. tuberculosis* with a novel mechanism of protein-protein interaction was verified with further studies here.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cloning

M. smegmatis mc^2 155 was obtained from ATCC and cultured in 7H9 broth or LB-Tween 80 (0.1%). *M. tuberculosis* H37Rv genomic DNA and whole cell lysates were obtained from BEI resources. A plasmid, pMsmTopoI, for overexpression of

topoisomerase I in *M. smegmatis* was constructed by placing *M. smegmatis topA* coding sequence (amplified with primers shown in Table 3. T2) under the control of the tetracycline inducible TetRO promoter in pKW08 vector (Williams et al., 2010) via Gibson assembly cloning (Gibson, 2011). Plasmid pMsmTopoI-CTD was then constructed for overexpression of the C-terminal domains D5-D8 plus tail (residues 627-936) by deleting the N-terminal domains D1-D4 (residues 1-626) via site-directed mutagenesis. Overexpression of *M. smegmatis* topoisomerase I (MsmTopoI) in *E. coli* BL21 STAR DE3 (Invitrogen) was achieved by Gibson cloning MsmtopA gene into pET His6 Mocr TEV LIC cloning vector (2O-T, Addgene Plasmid# 29710). The vector was later used for constructing various subdomains of topoisomerase I via site directed mutagenesis (Table 3. T3). Cloning of recombinant *M. tuberculosis* topoisomerase I (MtbTopoI) and its CTD in vector pLIC-HK (Doyle, 2005) for expression in *E. coli* BL21- CodonPlus(DE3)-RP (Agilent) was described previously (Annamalai et al., 2009). *M. smegmatis* RNAP beta (RpoB) and beta' (RpoC) subunits were inserted by Gibson cloning into the SspI site of pLIC-HK, and transformed into *E. coli* SoluBL21 (Genlantis) cells for protein expression and purification. The PCR primers used in the cloning, and site-directed mutagenesis are listed in the Tables 3. T2 and 3. T3.

Table 3. T1. Strains used in this study

Strain	Description	Source
MS_WT	Wild type *M. smegmatis* mc^2 155	ATCC
H37Rv	*M. tuberculosis*	ATCC
MS_CTD TopA	*M. smegmatis* transformed with a vector pKW08_CTD TopA. This strain over-expresses the C-terminal domain of topoisomerase I, upon induction with tetracycline. Western blots have confirmed that the over-expression is tightly regulated.	This study
MS_NOLUX	*M. smegmatis* transformed with a vector pKW08. This strain was used as a control in the *in vivo* stress experiments.	Williams et al., 2010

Table 3. T2. PCR primers for Gibson cloning

Primer	Gene	Sequence (5'-3')
RNA Polymerase beta_LIC_FP	rpoB	CTGTACTTCCAATCCAATGTGCTGGAAGG ATGCA
RNA Polymerase beta_LIC_RP	rpoB	ATCCGTTATCCACTTCCAATCTACGCGAGA TCCTCGAC
RNA Polymerase beta'_LIC_FP	rpoC	CTGTACTTCCAATCCAATGTGCTAGACGT CAACTTC
RNA Polymerase beta'_LIC_RP	rpoC	CGTTATCCACTTCCAATTTAGCGGTAATCC GAGTAG
MsmTopoI_pKW08_FP	MsmtopA	TTCGCGGATCCTTGGCTGGCGGCG ACCGCGG
MsmTopoI_pKW08_RP	MsmtopA	TTCTCAAGCTTCTAGGCCTTC TTGGCGGCGG
MsmTopoI_2OT_FP	MsmtopA	GGGATCGAGGAAAACCTGTACTTCCAAATG GCTGGCGGCGACCG
MsmTopoI_2OT_RP	MsmtopA	GCGGATCCGTTATCCACTTCCAATATTGTTC GGCGGAAACCTAGGCCTTCTT
D1-D8_MsmTopoI_2OT_FP	D1-D8 MsmtopA	GGGATCGAGGAAAACCTGTACTTCCAAATG GCTGGCGGCGACCG
D1-D8_MsmTopoI_2OT_RP	D1-D8 MsmtopA	GCGGATCCGTTATCCACTTCCAATATTGTTA GGCACGGCGGTCGG

Table 3. T3. Primers used for Site-Directed Mutagenesis

Primers for Site-Directed Mutagenesis		
Primer	**Sequence (5'-3')**	**Description**
CTD-MsmTopoI_ pKW08_FP	GGATCCTGTCAGGATTCCAC GATGAGAG	Deletion of the N-terminal domain from the full length MsmTopA gene, that was previously cloned into pKW08.
CTD-MsmTopoI_ pKW08_RP	GGCGTCGAGGGTTCGATCGC G	
NTD-MsmTopoI_2OT_FP	TAGAACCTCGAAGGCATCGA CGC	Insertion of a stop codon in the MsmtopA gene for early termination at the end of the N-terminus.
NTD-MsmTopoI_2OT_RP	GCCGCCGACGAGCTGCTT	
D1-D5_MsmTopoI_2OT_FP	GCGTCCCTCTTGCTATGTGG CGAAGAGCTTTTCGG	The proline (702) of MsmtopoI was substituted with a stop codon. The resulting protein (1-701) is termed as D1-D5.
D1-D5_MsmTopoI_2OT_RP	CCGAAAAGCTCTTCGCCACA TAGCAAGAGGGACGC	
D1-D6_MsmTopoI_2OT_FP	GCCCACGACGCGCTACAGCG ACAGCAGC	The proline (786) of MsmtopoI was substituted with a stop codon. The resulting protein (1-785) is termed as D1-D6.
D1-D6_MsmTopoI_2OT_RP	GCTGCTGTCGCTGTAGCGCG TCGTGGGC	
D1-D7_MsmTopoI_2OT_FP	CGGCCACGGCGTTTCTACTC GGCGTAGATCTT	The proline (840) of MsmtopoI was substituted with a stop codon. The resulting protein (1-840) is termed as D1-D7.
D1-D7_MsmTopoI_2OT_RP	AAGATCTACGCCGAGTAGAA ACGCCGTGGCCG	

Protein expression and purification

DNA topoisomerase I, sub-domains of topoisomerase I, RNAP beta, and RNAP beta' subunits were purified as recombinant proteins for protein-protein interaction studies. Overexpression of these recombinant proteins in the *E. coli* hosts was induced with 1 mM IPTG. Following the overexpression and cell lysis, recombinant proteins were purified to near homogeneity by affinity chromatography using Ni-Sepharose 6 Fast flow beads (GE Healthcare Life Sciences) followed by size-exclusion chromatography.

Affinity Chromatography: The MsmTopoI over expression clone, BL21 AI-2OT-MsmTopA, is cultured to exponential phase of growth in LB-NaCl (0.5 M) medium. At the exponential phase, the cells were induced with 1mM IPTG, 0.2% L-Arabinose at 20°C for 12 hours. The cell culture was pelleted, and the cells were lysed by freeze-thaw cycles. The whole cell lysate was spun at 32000 rpm for 2 hours, and the supernatant soluble protein fraction was passed through Ni^{+2}-Sepharose fast flow column for the purification of the hexa-histidine tagged topoisomerase I. A wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole; pH: 8.0) was passed along the column to remove any non-specifically bound proteins. A gradient elution method was followed to elute the recombinant proteins bound to the column. The elution buffer has imidazole concentrations ranging from 20 mM - 400 mM. The eluate fractions having the desired protein was dialyzed into 20 mM KH_2PO_4, 1 mM EDTA, 0.1 M KCl, 10% Glycerol.

TEV Protease reaction (Fusion tag removal): The recombinant protein was treated with the TEV protease for the removal of the purification handle (6xHis-Mocr tag) (Phan et al., 2002) . 1 part of TEV protease, a highly sequence-specific cysteine protease from Tobbaco Etch Virus, and 25 parts of the recombinant protein (6xHis-Mocr-

MsmTopoI) were incubated together at 20°C for 4 hours initially, and then at 4°C overnight. The reaction mixture contains 50 mM Tris (pH: 8.0), 0.5 mM EDTA, and 1 mM DTT. The TEV protease reaction product was passed through the Ni^{+2}-Sepharose column, and the flow-through (MsmtopoI, devoid of tag) is collected. The elutions from the column were made with 400 mM imidazole containing phosphate buffer.

Gel-Filtration (Size Exclusion Chromatography): The flow-through from the TEV-protease reaction, the recombinant protein devoid of the tag, was passed through a gel-filtration column for a separation based on the size. A HiPrep 26/60 Sephacryl S-200HR column (GE Healthcare) was equilibrated with degassed Tris buffer (20 mM Tris; pH: 8.0, 0.3 M KCl). The sample (load) was passed through the column, and washes were carried out with Tris buffer. The eluate fractions containing the desired protein were pooled, and dialyzed into storage buffer (0.1 M KH_2PO_4, 0.2 mM EDTA, 0.2 mM DTT, 50% Glycerol). A similar approach was followed in the purification of all the other proteins that were a part of this study.

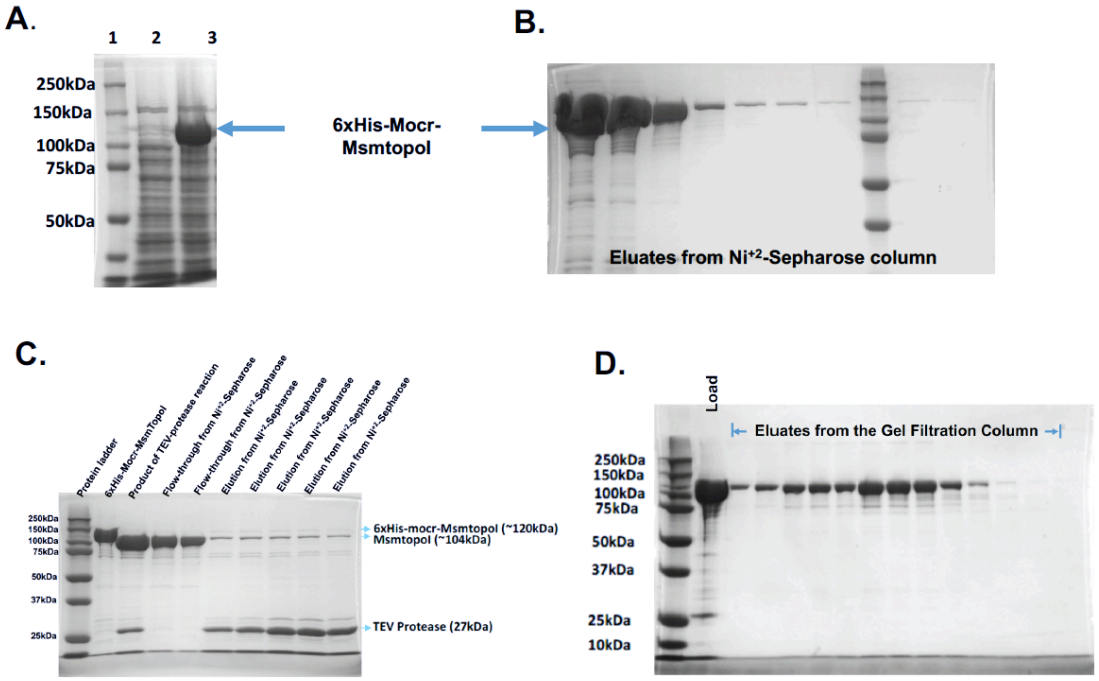


Figure 3.1. Protein (*M. smegmatis* topoisomerase I) purification; (A) Protein over-expression was confirmed. Lane 2, represents the whole cell lysate of BL21 AI-2OT-MsmTopA before the protein over-expression. Lane 3, represents the soluble protein fraction from the cells subjected to protein over-expression. (B) The eluates from the Ni^{+2}-Sepharose column. (C) The recombinant tag was removed by the TEV protease reaction. (D) The eluate fractions from the Gel filtration column were electrophoresed on a SDS-PAGE, and coomassie stained.

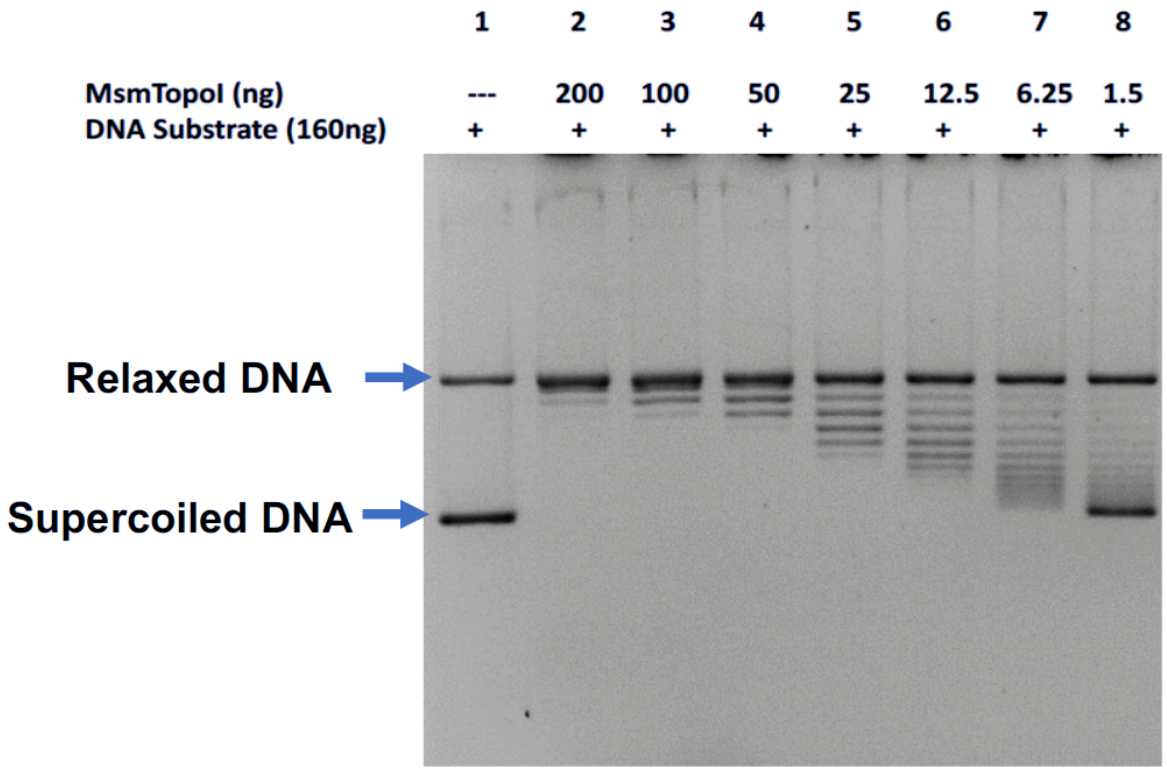


Figure 3.2. Relaxation activity of the purified topoisomerase I; The assay successfully demonstrates the relaxation of the negatively supercoiled DNA in the presence of the enzyme (Lane 2-8).

Table 3. T4. Recombinant Proteins used in the study

Protein	Organism	Cloning vector	Cleavable fusion tag (TEV Site)	Recombinant Tag (N-terminal)
Topoisomerase I (1-936 Amino acids)	*M. smegmatis*	2O-T	Yes	6xHis-Mocr
Topoisomerase I (1-934 Amino acids)	*M. tuberculosis*	2O-T	Yes	6xHis-Mocr
Topoisomerase I (D1-D8) (1-910 Amino acids)	*M. smegmatis*	2O-T	Yes	6xHis-Mocr
Topoisomerase I (D1-D7) (1-840 Amino acids)	*M. smegmatis*	2O-T	Yes	6xHis-Mocr
Topoisomerase I (D1-D6) (1-786)	*M. smegmatis*	2O-T	Yes	6xHis-Mocr
Topoisomerase I (D1-D5) (1-701 Amino acids)	*M. smegmatis*	2O-T	Yes	6xHis-Mocr
Topoisomerase I (D1-D4); N-terminal (1-626 Amino acids)	*M. smegmatis*	2O-T	Yes	6xHis-Mocr
Topoisomerase I (C-terminal) (657-936 Amino acids)	*M. smegmatis*	2O-T	No	6xHis
RNA Polymerase beta'	*M. smegmatis*	LIC-HK	Yes	6xHis
RNA Polymerase beta	*M. smegmatis*	LIC-HK	Yes	6xHis

Table 3. T4 contains a comprehensive list of proteins that were expressed, and purified for this study. Mocr, a fusion tag, enhances the solubility of the recombinant protein (DelProposto et al., 2009).

Purification of *M. tuberculosis* topoisomerase IgG from the Rabbit Serum

Purified antibodies were used in the Co-Immunoprecipitation (Co-IP) assays. The antibodies were purified from the Rabbit serum obtained before and after injection with *M. tuberculosis* topoisomerase I (MtbTopoI). Antibody (IgG) purification was carried out using Promega Protein A magnetic beads (G8781), according to the manufacturer's protocol. Briefly, 100µl of protein A beads were initially equilibrated in wash buffer (8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.68 mM KCl; pH: 7.4), and incubated with 250 µl of the serum for 4 hours at 4°C. Magnetic field was applied by placing the reaction tubes in a Magnetic separation stand. The unbound components of the serum (supernatant) were carefully removed, and the bead pellet was washed thrice in the wash buffer. Finally, the bead pellet was eluted with 150µl of 100 mM Glycine-HCl (pH: 2.7), and the eluate was neutralized by mixing with 30 µl of 2 M Tris buffer (pH: 7.5). The concentrations of the pure antibodies were verified based on a colorimetric, Bradford dye-binding method (Bio-Rad Protein Assay Kit; 5000006).

Figure 3.3. Cross-reactivity of Rabbit MtbTopoI polyclonal antibodies with MsmTopoI; Rabbit Polyclonal antibodies that were generated against MtbTopoI can cross-react with MsmTopoI due to a high sequence homology between these proteins. Western blotting of the soluble lysates from *M. smegmatis* mc^2 155 was carried out to verify the cross-reactivity of the antibody. Lane 1: Soluble lysate (10 µg) from H37Rv. Lane 2-4: Purified MtbTopoI (25 ng, 50 ng, 75 ng). Lane 5: Purified MsmTopoI (25 ng). Lane 6-8: Soluble lysates (10 µg) of *M. smegmatis* mc^2 155 over the course of its growth (Lane 6: OD_{600}- 0.8, Lane 7: OD_{600}- 1.5, Lane 8: OD_{600}- 3.0).

Co-Immunoprecipitation (Co-IP)/ Tandem Mass spectrometry

The protein networking of *M. smegmatis* topoisomerase I was analyzed by Co-Immunoprecipitation coupled to tandem mass spectrometry (Free et al., 2009). Briefly, stationary phase *M. smegmatis* mc^2 155 pellet was suspended in a lysis/wash buffer (50mM NaCl, 50 mM NaH_2PO_4, pH 8.0, 0.1% NP-40), and then subjected to sonication (ultrasound energy) for releasing the cellular contents. The lysate centrifuged at 16000xg for an hour, and the supernatant soluble lysate was used as prey in the Co-IP assays. Protein A/G plus agarose beads (Santa Cruz) were used for the Co-IP assays. Each Co-IP reaction contained 500 µg of the total soluble protein, 10 µg of the purified antibody, and the Protein A/G plus agarose beads. The eluates from the reaction were subjected to SDS-PAGE electrophoresis, and the resolved protein bands were excised for characterization of the proteins by mass spectrometry. A detailed protocol is provided in Chapter 2.

Pull-down assay/ Tandem Mass spectrometry

The protein networking of *M. smegmatis* topoisomerase I was also verified by a Pull-down approach coupled with Mass spectrometry (Brymora et al., 2004). In this approach, the target protein was used in concentrations higher than usually found in the physiological conditions. High concentration of target protein, recombinant MsmTopoI (N-terminal 6xHistidine tagged), is incubated with *M. smegmatis* mc^2 155 soluble cell extract for 2 hours at 4°C, and later immobilized on a HisPur Cobalt Agarose resin (Thermofisher). The wash, elution, SDS-PAGE electrophoresis, and analysis by mass spectrometry is described in detail in chapter 2.

Reverse Pull-down assay/ Immunoblotting

A reverse pull-down assay was used to validate the protein interactions identified by pull-down assay. In this assay, a recombinant *M. smegmatis* RNAP beta' subunit (N-terminal 6xHistdine) was used as a bait to pull-down topoisomerase I from the soluble cell extract. The Pull-down protocol is similar that described in the previous section. Western blot analysis with rabbit polyclonal antibodies against MtbTopoI was used to detect the presence of topoisomerase I in the eluate.

Pull-down, Co-IP assays on *M. tuberculosis* (H37Rv) cell extract

The conservation of RNA Polymerase-topoisomerase I protein-protein interactions in *M. tuberculosis* was verified by pull-down, and Co-IP assays. Whole cell extract of *M. tuberculosis* H37Rv was provided by BEI Resources. The extract was spun at 16000xg for 20 min at 4^{o}C, and the soluble protein fraction was used for the assays. Recombinant MtbTopoI with N-terminal 6x-Histidine was used in the pull-down assay, while antibody against topoisomerase I was used in the Co-IP assay as a bait to determine if RNA polymerase could be captured from the cell extract. Following SDS PAGE, the proteins in the eluates from the assays were immunoblotted with a monoclonal antibody against RNA Polymerase beta (Biolegend) which can recognize the RNAP beta subunits (Bergendahl et al., 2003) across multiple species including *M. tuberculosis* and *M. smegmatis*.

Physical interactions between RNA Polymerase beta' subunit and Topoisomerase I

The use of cell extract, as prey, in the pull-down and Co-IP assays can only provide information on the protein networking of a target protein. The physical interactions, if any,

between two proteins can be studied by use of pure proteins (as bait and prey) in the pull-down (Banda et al., 2016), and Co-IP assays. Isothermal Calorimetry, Surface Plasmon Resonance could be used for obtaining the kinetic parameters of the physical interaction between proteins (Pierce et al., 1999; Tiwari et al., 2016). However, these methods require large amounts of protein, and are expensive. Hence, the physical interactions between topoisomerase I, and RNA polymerase β,β' subunits were studied by minor modifications of pull-down, and Co-IP assays. The fusion tag [6xHis or 6xHis-mocr] of purified prey proteins was cleaved prior to their use in the assay (Phan et al., 2002).

Effect of *M. smegmatis* TopoI-CTD overexpression on sensitivity to antibiotics or hydrogen peroxide

M. smegmatis strain over expressing the CTD of topoisomerase I from plasmid pMsmTopA-CTD, or a control *M. smegmatis* strain transformed with the cloning vector, were cultured to stationary phase in 7H9 medium containing Hygromycin (100µg/ml), and Tetracycline (25ng/ml). The cultures were adjusted to OD_{600}=1.0 in 7H9 medium, and 200µl of the OD adjusted culture was spread on a LB plate. Antibiotic or H_2O_2 was applied to paper discs placed at the center of the plates, followed by incubation at 37°C for 60 hrs. The diameter of the zone of inhibition was measured. For comparison of viable colony counts following stress challenge, the *M. smegmatis* strains were grown in Luria broth-Tween 80 (0.1%) containing Hygromycin (100µg/ml) and Tetracycline (25ng/ml). At exponential phase, the cultures were treated with Moxifloxacin (0.5 µM or 1 µM) for 12 hours at 37°C. The treated cultures, and untreated cultures were serially diluted, and plated

on LB-Agar. The viable colonies were counted, and the relative viability ratio (treated versus untreated) was calculated. Similarly, viable colony counts following treatment with hydrogen peroxide (10 mM or 20 mM) was analyzed with the strains cultured in 7H9 medium containing Hygromycin (100 μg/ml), and Tetracycline (25 ng/ml).

RESULTS

Identification of RNA Polymerase as protein-protein interaction partner of MsmTopoI

Protein interaction network of the target protein (MsmTopoI) was first analyzed by Co-IP (Figure 2.2) coupled to Mass spectrometry. Following SDS-PAGE of the immunopreciptated proteins, coomassie-stained proteins in the bands, labeled as a-d (lane 5 of figure 2.2, B), were selected as *M. smegmatis* proteins co-immunopreciptated by MtbTopoI antibodies that can also recognize MsmTopoI (Figure 3.3). The control purified pre-immune antibodies did not precipitate any proteins corresponding to the bands a-d, as evident from the coomassie stain (Lane 4 of figure 2.2, B). LC-MS/MS for identification of proteins in the bands, a-d, of the MtbTopoI Co-IP reaction and the corresponding bands in the control reaction was carried out. A comprehensive list of proteins precipitated by MtbTopoI IgG alone, and not the control pre-immune IgG is provided in the table 2. T2. The ability of the antibodies raised against MtbTopoI to immunoprecipitate MsmTopoI from the cell lysate was confirmed by western blot analysis of the Co-IP eluates (Figure 2.2, C). LC MS/MS analysis identified *M. smegmatis* RNAP beta and beta' subunits in the protein band "a".

Protein networking of MsmTopoI was further investigated by Pull-down assay as described in Figure 2.3, A with His-tagged MsmTopoI and MsmTopoI-CTD. Coomassie-stained proteins (e, f in lanes 7 and 8 of Figure 2.3, B) of *M. smegmatis* pulled down by either His-tagged MsmTopoI or MsmTopoI-CTD, but not control His-Mocr (lane 6, Figure 2.3, B) were analyzed by LC MS/MS. *M. smegmatis* RNA polymerase beta and beta' subunits were again identified in protein band "e".

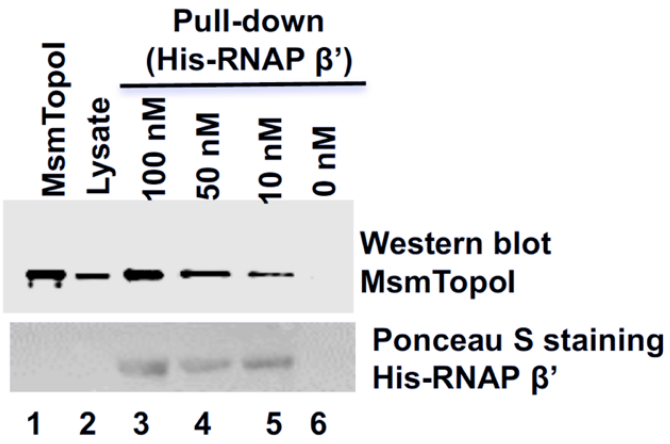


Figure 3.4. Reverse pull-down of MsmTopoI from *M. smegmatis* soluble lysate with purified recombinant RNA polymerase β' subunit (N-terminal His-tagged). Following SDS PAGE, the proteins eluted from the HisPur Cobalt were analyzed by Western blot using anti-topoisomerase I antibodies. Lane 1: purified recombinant MsmTopo I (100 ng). Lane 2: 15 µg of total proteins in *M. smegmatis* soluble lysate. Lanes 3-6: Eluates from Pull-down assay using 500 µg of total proteins in *M. smegmatis* soluble lysate with His-tagged RNAP β' subunit of concentrations 100nM (Lane 3), 50 nM (Lane 4), 10 nM (Lane 5); 0 nM (Lane 6). The nitrocellulose membrane was also stained with Ponceau S for detection of the recombinant bait (His-tagged RNAP β') used in the assay.

Protein-protein interaction between RNA polymerase and topoisomerase I in *M. smegmatis* was further verified by reverse pull-down followed by immunoblotting (Figure 3.4). In this assay, recombinant RNA polymerase beta' subunit (N-terminal 6xHis) was used a bait to capture topoisomerase I from the *M. smegmatis* soluble cell lysate. The assay

confirmed a concentration dependent binding between the two proteins (lane 3-5 in Figure 3.4). The combined results from Co-IP and Pull-down experiments confirmed the networking of topoisomerase I and RNAP with a novel mechanism that does not involve the zinc ribbon domains found in other bacterial topoisomerase I.

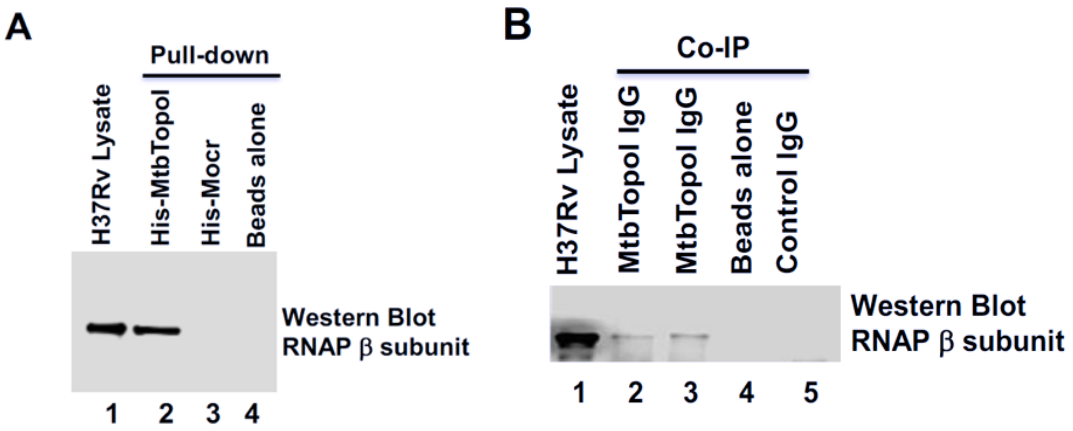


Figure 3.5. *M. tuberculosis* H37Rv topoisomerase I and RNA polymerase are protein-protein interaction partners. *M. tuberculosis* RNA polymerase (RNAP) was detected by western blot using a monoclonal antibody against *E. coli* RNAP β subunit that cross-react with mycobacteria RNAP. (A) Pull-down assay. Lane 1: Total soluble lysate of *M. tuberculosis* H37Rv (10 µg). Eluates from HisPur cobalt resin incubated with 250 µg of lysate and purified His-tagged MtbTopoI (lane 2), 6xHisMocr (lane 3) or without any His-tagged protein (lane 4). (B) Co-Immunoprecipitation assay. Lane 1: Total soluble lysate of *M. tuberculosis* H37Rv (10 µg). Immunoprecipitates from 250 µg of total soluble lysates incubated with IgG against MtbTopoI (lanes 2,3), no IgG added (lane 4), IgG from pre-immune serum (lane 5) were analyzed by western blot with the antibody recognizing RNAP β subunit.

RNAP-Topoisomerase I interactions are conserved in *M. tuberculosis* H37Rv

M. smegmatis is a useful model system for the understanding of processes in pathogenic *M. tuberculosis* (Shiloh et al., 2010). The protein-protein interaction between

topoisomerase I and RNAP could be conserved among these mycobacteria. The RNAP-topoisomerase I interaction in *M. tuberculosis* H37Rv was studied by Pull-down (Figure 3.5, A), and Co-IP assays (Figure 3.5, B). Results from both approaches (lane 2 in Figure 3.5, A; lanes 2,3 in Figure 3.5, B) confirmed the conservation of RNAP-topoisomerase I interaction in *M. tuberculosis*.

Direct physical interaction between Topoisomerase I and RNA polymerase beta' subunit

The results from the Co-IP and pull-down experiments indicate the presence of Mycobacteria topoisomerase I and RNAP in the same complex. However, a direct physical interaction between these two partners in the absence of DNA or other Mycobacteria proteins was not addressed. The direct protein-protein interactions were analyzed with purified recombinant proteins. Physical interaction between the *M. smegmatis* RNA polymerase beta' subunit and topoisomerase I was demonstrated by both co-immunoprecipitation (Figure 3.6, A) and pull-down experiments (Figure 3.6, B). This protein-protein interaction is specific. No interaction with MsmTopoI could be observed for *M. smegmatis* RNA polymerase beta subunit (Figure 3.6, A). *E. coli* topoisomerase I, with CTD that share no homology with MsmTopoI, also did not interact with *M. smegmatis* RNA polymerase beta' subunit (Figure 3.6, C).

Mapping of MsmTopoI sequence required for interaction with RNAP

Pull-down (Figure 3.7, A) and Co-IP (Figure 3.7, B) assays were first conducted to verify that only the CTD (D5-D8, plus tail at C-terminal end), but not the NTD (D1-D4)

of MsmTopoI is involved in the protein-protein interaction with RNAP. Pull-down assay with purified recombinant His-tagged proteins retaining different regions of the CTD of MsmTopoI as bait was then used to further identify sequence in MsmTopoI-CTD required for pull-down of RNAP (prey) from the soluble cell extract. The pull-down of RNAP (prey) by protein-protein interaction could be observed only when the full-length MsmTopoI, or MsmTopoI-CTD was used as the bait (Figure 3.8, A; Lane 2,8). Equal molar amounts of the bait protein (subdomains of topoisomerase I) was used in these assays. MsmTopoI-909t (D1-D8) of topoisomerase I is only missing the tail at the C-terminal end (last 27 amino acids), but it still lacks affinity for RNAP. This data indicated that the protein-protein interaction with RNAP is mediated via the C-terminal tail sequence (amino acids 910-936) of MsmTopoI (Figure 3.8, B).

Increased sensitivity to antibiotics and oxidative stress from overexpression of MsmTopoI-CTD

Overexpression of the MsmTopoI-CTD is expected to inhibit the interaction between MsmTopoI and RNAP. Following tetracycline-induced overexpression of recombinant MsmTopoI-CTD in *M. smegmatis* mc2 155 (Figure 3.9, A), less full length MsmTopoI was pulled down by His-tagged RNAP beta' subunit (Figure 3.9, B). The MsmTopoI-CTD overexpression did not affect the growth rate in 7H9 media (Figure 3.10). However, increased sensitivity to stress challenge from antibiotics (moxifloxacin, streptomycin) and hydrogen peroxide resulted from the MsmTopoI-CTD overexpression, as seen from the comparison of the diameters of zone of inhibition between the control strain and MsmTopo-CTD overexpressing strain (Figure 3.11, B). Following treatment

with either moxifloxacin (0.5 μM, 1 μM) or hydrogen peroxide (10 mM, 20 mM), there was significantly greater loss of viability for the MsmTopoI-CTD overexpressing strain (Figure 3.11, A). These results showed that inhibition of MsmTopoI-CTD protein-protein interactions can be correlated to enhanced sensitivity to antibiotics and oxidative stress.

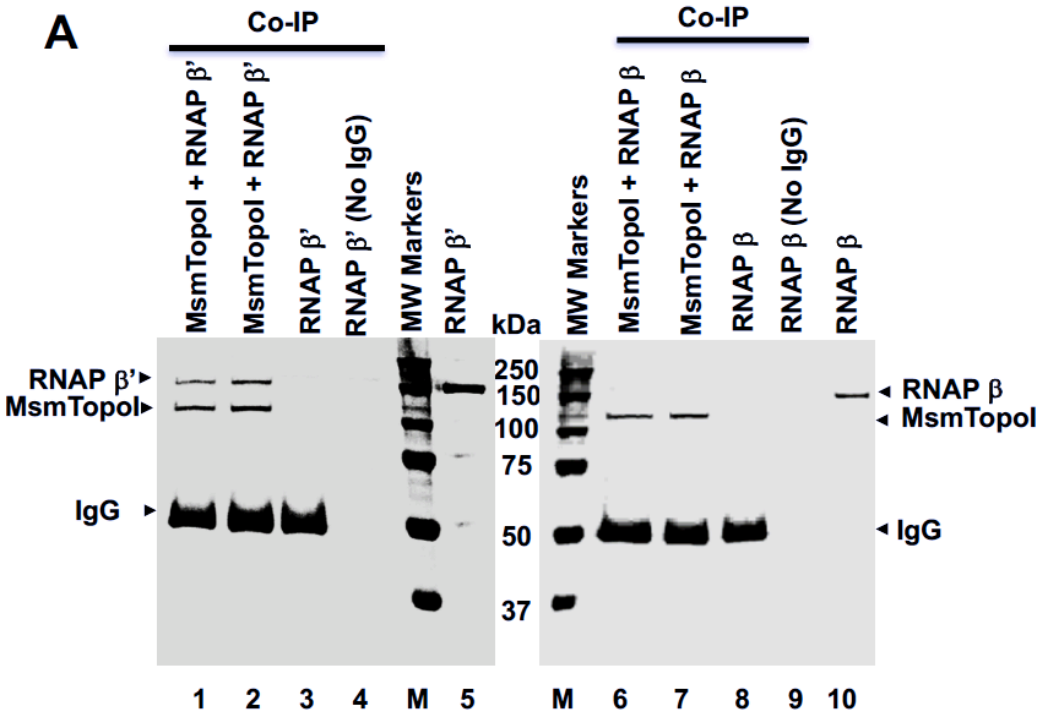
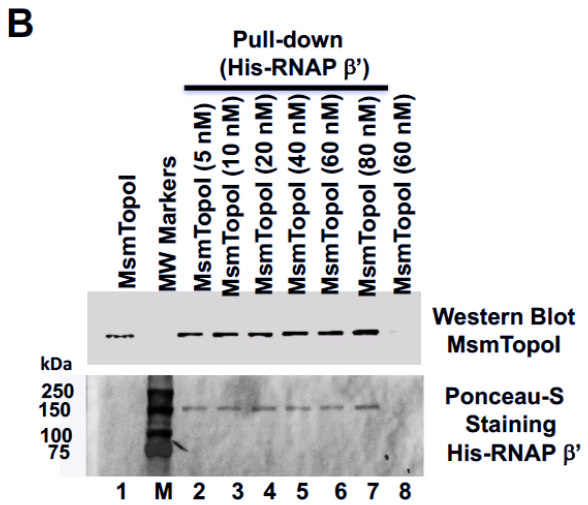


Figure 3.6. Direct physical interaction between purified *M. smegmatis* topoisomerase I and RNA polymerase β' subunit. (A) Purified RNA polymerase β' subunit (lanes 1-4) but not β subunit (lanes 6-9) can be co-immunoprecipitated with MsmTopoI. The co-immunoprecipitation reaction contained 100 (lanes 1, 6) or 200 nM of MsmTopo I (lanes 2, 7) along with 125 nM of β' (lanes 1-4) or β (lanes 6-9) subunit of RNAP. Lanes 3, 8: no MsmTopoI added. Lanes 4, 9: no IgG added. Lanes 5, 10: purified RNAP subunit (1.5 µg). The proteins in the gel were stained with Coomassie blue following SDS PAGE. M: Molecular weight standards.



C

	1	2	3	4	5	6	7	8
M. smegmatis RNAP β' (15nM)	---	---	+	+	+	---	---	---
M. smegmatis RNAP β ; (15nM)	---	---	---	---	---	+	+	+
E. coli Topoisomerase I (nM)	---	20	5	10	20	5	10	20
E. coli Topoisomerase I (25ng)	+	---	---	---	---	---	---	---
HisPur cobalt resin	---	+	+	+	+	+	+	+

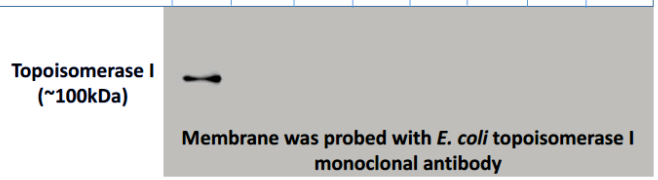


Figure 3.6. Direct physical interaction between purified *M. smegmatis* topoisomerase I and RNA polymerase β' subunit
(B) Pull-down assay with HisPur cobalt resin. Lane 1: 25 ng of MsmTopoI. The pull-down reactions contained 15 nM of His-RNAP β' subunit incubated with 5, 10, 20, 40, 60, 80 nM (lanes 2-7) of MsmTopoI. Lane 8: control of 60 nM MsmTopoI with no RNAP β' subunit added. MsmTopoI pulled down by His-RNAP β' subunit were visualized by western blot. The nitrocellulose membrane was then stained with Ponceau S. M: Protein molecular weight standards.
(C) Pull-down assay for cross-interaction studies; Lanes 3, 4, 5 are loaded with eluates from the pull-down reactions of recombinant RNA polymerase β' subunit. Lanes 6, 7, 8 represent the eluates from the pull-down with recombinant RNA polymerase β.

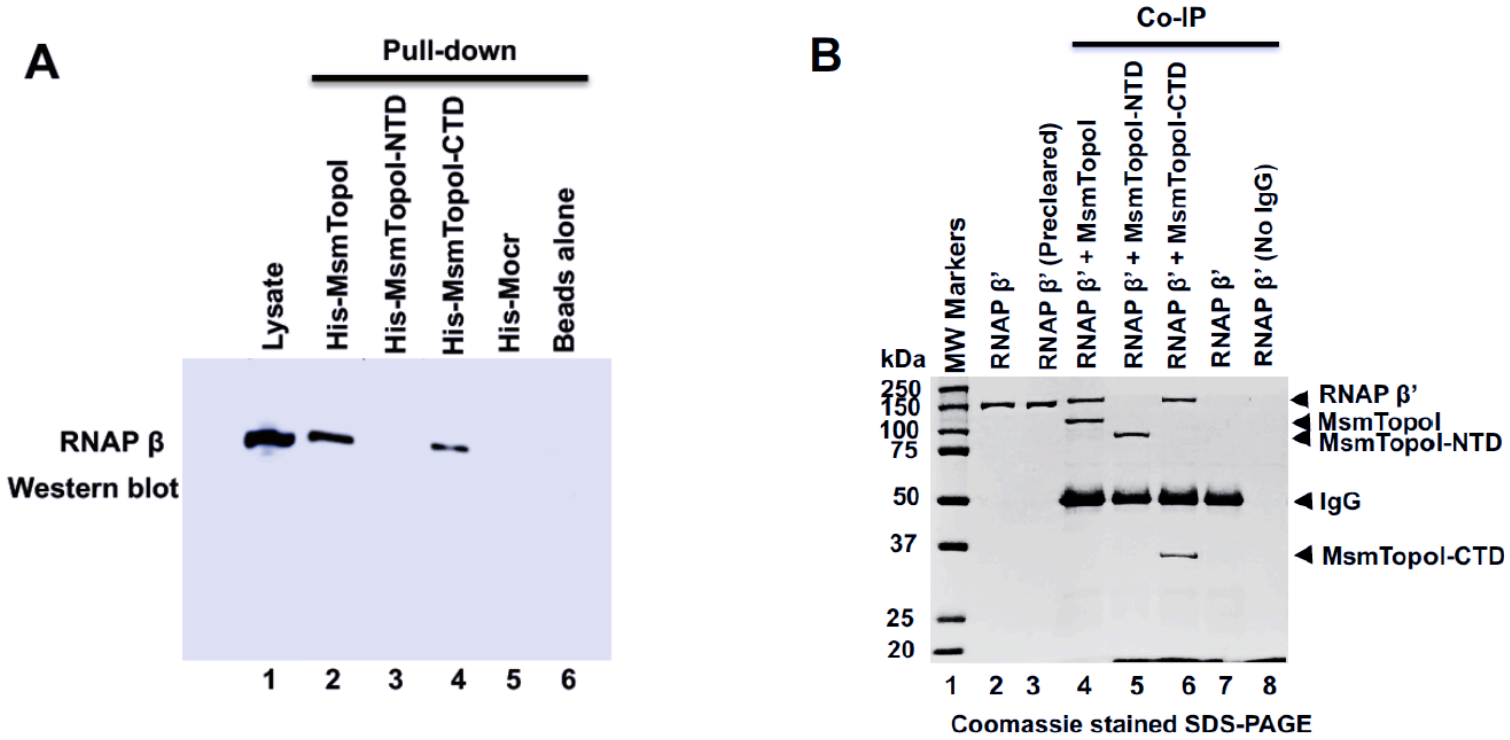


Figure 3.7. Identification of MsmTopoI domain required for interaction with RNAP.
(A) *M. smegmatis* soluble lysate was incubated with the His-tagged recombinant MsmTopoI or its fragment, and the eluates from the reaction were analyzed by western bloting. TopoI-CTD but not TopoI-NTD can interact with RNAP in *M. smegmatis* cell lysate. (B) Direct physical interaction between TopoI-CTD and RNAP β' subunit was verified by Co-IP assay. Purified RNAP β' subunit was incubated with MsmTopoI or its fragment, and the proteins immunoprecipiated by TopoI antibodies from the reactions were analyzed by SDS-PAGE/Coomassie staining. The assay confirms a physical interaction of RNAP β' with MsmTopoI-CTD (Lane 6), and not MsmTopoI-NTD (Lane 5). Purified RNAP β', by itself, did not bind with the antibody (Lane 7) or the beads (Lane 8).

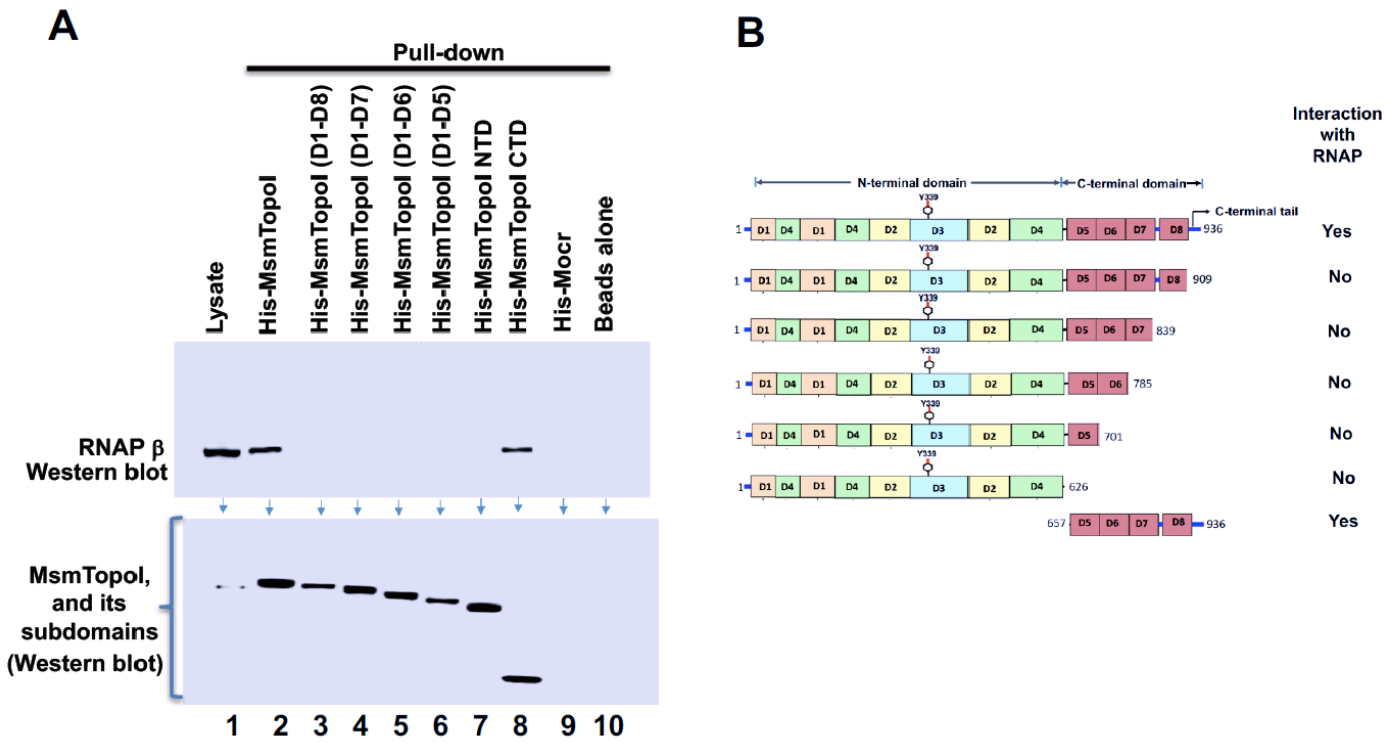


Figure 3.8. Mapping of MsmTopoI sequence required for interaction with RNAP.
(A) Pull-down assay with MsmTopoI truncation mutants lacking different segments of the TopoI-CTD. *M. smegmatis* soluble lysate was incubated with different constructs of the recombinant MsmTopoI, and the eluates were probed for the presence of RNAP. The full-length recombinant MsmTopoI (Lane 2), and CTD-MsmTopoI (Lane 8) can interact with RNAP.
(B) Domain arrangement of MsmTopoI is shown here. The results from pull-down and Co-IP assays, taken together, demonstrate that the tail at the C-terminal end of MsmTopoI interacts with RNAP.

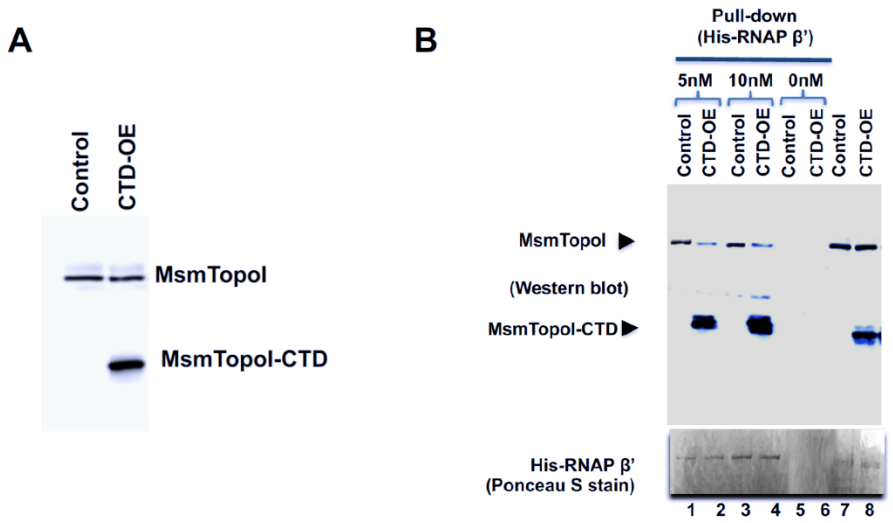


Figure 3.9. Inhibition of MsmTopoI-RNAP interaction by overexpression of recombinant MsmTopoI-CTD.
(A) The tetracycline induced overexpression of MsmTopoI-CTD was confirmed by western blot analysis with rabbit polyclonal antibodies. Lane 1: Lysate (10 µg) of *M. smegmatis* transformed with control vector. Lane 2: Lysate (10 µg) of *M. smegmatis* transformed with pMsmTopoI-CTD.
(B) Pull-down of MsmTopoI from *M. smegmatis* lysate by His-tagged RNAP β' subunit is reduced by the competing, overexpressed MsmTopoI-CTD. Pull-down of MsmTopoI, and MsmtopoI-CTD from the lysate (350 µg) by His-RNAP β' (5 nM or 10 nM) was analyzed by western blot. Lane 1, 3: Pull-down reactions with lysate from the strain transformed with the control vector (control) in the presence of 5 nM His-RNAP β' or 10 nM His-RNAP β'. Lane 2, 4: Pull-down reactions with lysate from the strain overexpressing the MsmTopoI-CTD (CTD-OE) in the presence of 5 nM His-RNAP β' or 10 nM His-RNAP β'. Lane 5, 6: The lysates from either the control strain or the overexpression strain were incubated with the beads, as a negative control for the pull-down assays. Lane 7: lysate (10 µg) from *M. smegmatis* transformed with the control vector. Lane 8: lysate (10 µg) from *M. smegmatis* overexpressing the MsmTopoI-CTD. The nitrocellulose membrane was later stained with Poneau S for the detection of the Bait (His-RNAP β').

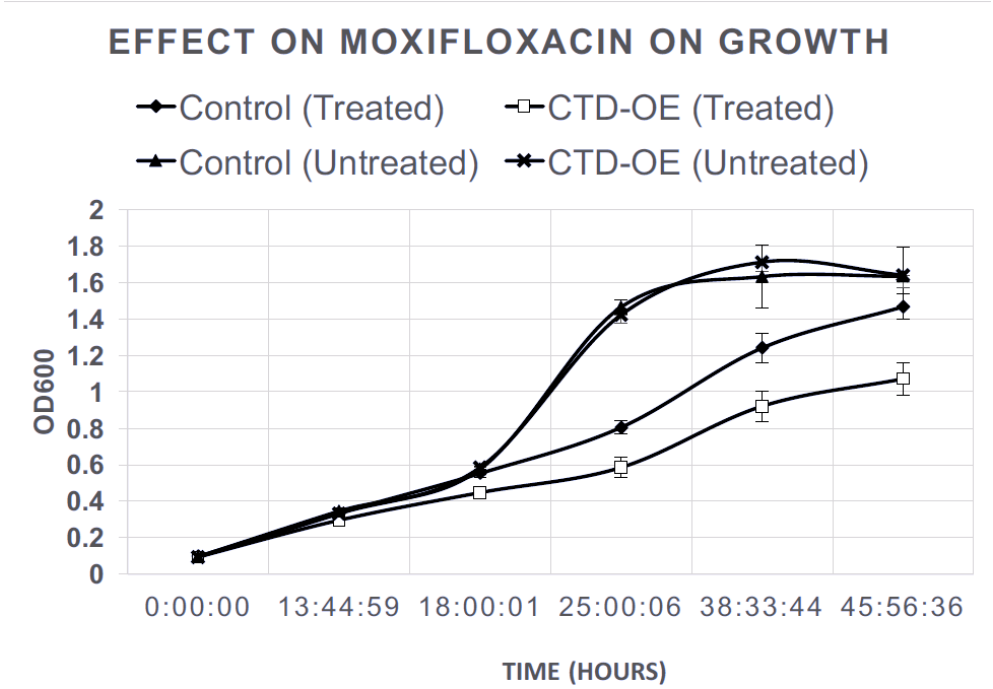


Figure 3.10. Effect of overexpressing the CTD of MsmTopoI on growth
The effect of overexpressing the CTD of MsmTopoI in *M. smegmatis* (CTD-OE) on its growth was monitored by measuring the OD_{600} over a period of 2 days. The overexpression by itself did not have any effect on growth pattern, as evident from the growth curves of the untreated cultures of the control and the overexpression strain. However, treating the cultures with a sub-MIC concentration of Moxifloxacin (0.15 μM) has slowed down the growth of the overexpression strain.

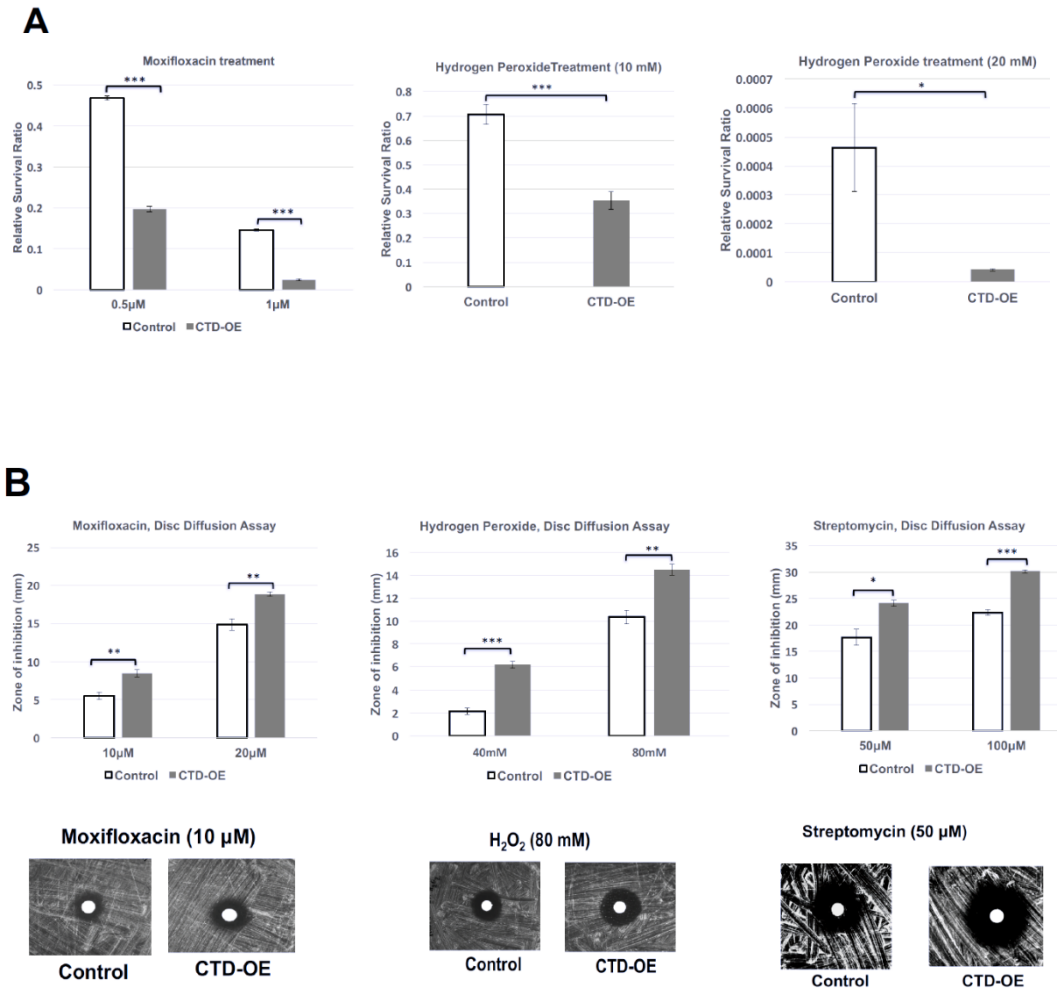


Figure 3.11. Effect of TopoI-CTD overexpression on sensitivity of *M. smegmatis* to stress challenge.

(A) Following treatment with moxifloxacin or hydrogen peroxide for 12 hours, the untreated and treated cultures of the vector control strain (control), and MsmTopoI CTD-overexpression (CTD-OE) strain were serially diluted, and plated on LB-Agar. The viable colony counts (CFU/ml) were determined to calculate the relative survival ratios as the colony counts of the treated cultures divided by the colony counts of the untreated cultures. (B) The tetracycline-induced cultures of the vector control strain (control), and the MsmTopo CTD-overexpression strain (CTD-OE) were spread on LB plates, and a blank paper disc was placed on the plate. 20 µl of the antibiotic or hydrogen peroxide was added to the disc. The plates were incubated at 37°C for 60 hours, and the zone of inhibition was measured. Error bars represent the standard deviation (n=3). Student's t-test was used to calculate the p-values (* $p<0.05$; ** $p<0.005$, ***$p<0.0005$).

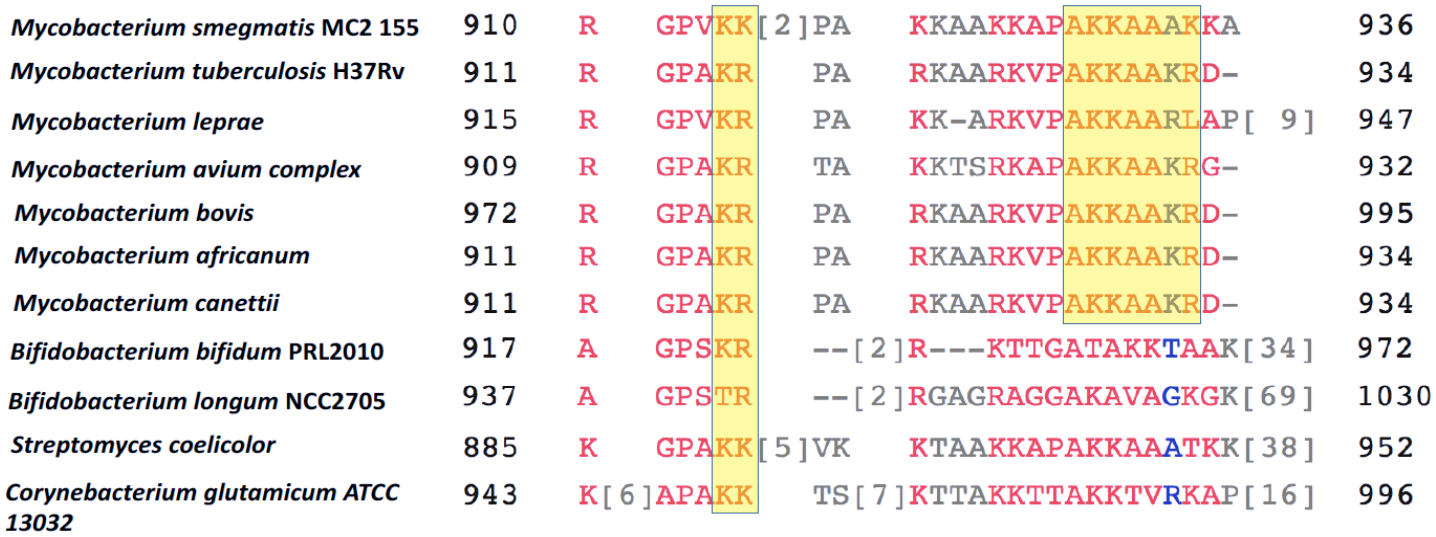


Figure 3.12. The C-terminal tail of topoisomerase I from actinobacteria is rich in basic amino acids
Topoisomerase I from different Actinobacteria were aligned using Constraint-based multiple alignment tool, COBALT (Papadopoulos & Agarwala, 2007). The regions of high conservation are highlighted. Mycobacterial topoisomerase I has a highly-conserved region (AKKAAAK) in the tail.

DISCUSSION

TopoI is the only type IA topoisomerase encoded in the genomes of Mycobacteria, and has been shown to be essential for the viability of both *M. tuberculosis* (Ahmed et al., 2014; Ravishankar et al., 2015) and *M. smegmatis* (Ahmed et al., 2015). The identification of the protein-protein interaction partners of Mycobacteria TopoI is likely to provide insights into the *in vivo* functions and regulation of this potential antibacterial target. It has been reported that the relaxation activity of MtbTopoI and MsmTopoI can be inhibited by interaction with a MazF homolog (Huang et al., 2010), as well as D-ribokinase (Yang et al., 2011). The catalytic activity of MtbTopoI can also be modulated by interaction with the nucleoid associated protein HU (Ghosh et al., 2014). These previously identified protein-protein interactions may be relevant for the regulation of Mycobacteria TopoI activity. However, experimental data on protein-protein interactions of Mycobacteria TopoI that may inform on the physiological setting of its function is currently not available. In this study, we tried to identify such potential partners present in total soluble proteins of *M. smegmatis* with the approaches of Co-IP and pull-down assays coupled to mass spectrometry.

RNA Polymerase was identified as a protein-protein interaction partner for MsmTopoI by both approaches. A recent ChIP-Seq study showed that *M. tuberculosis* TopoI and gyrase are recruited to genomic loci with high transcriptional activity, and MtbTopoI was localized behind RNAP to be in position for relaxing the negative supercoils generated during transcription (Ahmed et al., 2017). Our studies here demonstrated that *M. tuberculosis* RNAP can recruit TopoI via direct protein-protein interaction to facilitate the co-localization during transcription elongation. While protein-protein interaction with

the RNAP beta' subunit has been previously reported for the CTD of *E. coli* TopoI (Cheng et al., 2003), it should be noted that the CTD of mycobacteria TopoI share no sequence and structural similarity with the CTD of bacteria outside the Actinobacteria phylum. The CTD of *E. coli* TopoI are formed by zinc ribbon motifs stabilized by Zn(II) coordinated with four cysteines in each motif (D5-D7), or zinc ribbon like motifs of similar structures (D8-D9). Interaction sites for RNAP have been mapped to *E. coli* TopoI CTD regions of D5-D7 as well as D8-D9 (Cheng et al., 2003). Analysis of MsmTopoI protein sequence (Bhaduri et al., 1998) and determination of D1-D6 structure of MtbTopoI (Tan et al., 2016) have shown that the CTD of mycobacteria are formed by repeats of a distinctively different structural motif containing a beta sheet and alpha helix, with no Zn(II) present. The results reported here demonstrate that while the different bacterial species have evolved to have distinct sequence and structures of CTD in TopoI potentially through acquiring different duplicated gene segments during evolution, the protein-protein interaction with the beta' subunit of RNAP has simultaneously co-evolved through divergent mechanisms in different bacterial phylum.

Molecular simulations predicted that salt bridges and hydrogen bonds formed by basic residues positioned over a large molecular surface formed by the zinc ribbon motifs of *E. coli* TopoI are responsible for interactions with acidic residues in RNAP (Tiwari et al., 2016). In contrast, a novel mechanism utilizing a short stretch of C-terminal tail is employed instead for the TopoI-RNAP interaction in bacteria that do not have Zn(II) binding TopoI-CTD. As shown in Figure 3.12, the amino acid sequence of the C-terminal tail is highly conserved in mycobacteria TopoI sequence and rich in basic residues. The basic region represented by the C-terminal tail of MsmTopoI and MtbTopoI has been

proposed to participate in the binding of DNA to promote strand passage during catalysis (Ahmed et al., 2013). Direct interaction of the C-terminal tail with RNAP would facilitate the rewinding of single-stranded DNA following their exit from the RNAP elongation complex to prevent to prevent stabilization of R-loop structure and inhibition of transcription elongation. The TopoI-RNAP interaction may be especially important for efficient transcription response to stress conditions to achieve maximal survival following antibiotic or oxidative challenge. Competition for RNAP interaction by overexpressed recombinant MsmTopoI-CTD may have contributed to the increase in sensitivity to antibiotics and oxidative stress challenge observed here. Nevertheless, we cannot rule out that other protein-protein interactions inhibited by overexpressed MsmTopo-CTD may also contribute to the increased sensitivity. Other potential protein-protein interaction partners identified in our Co-IP and pull down experiments are currently being investigated in further studies.

TopoI is the only type IA topoisomerase in mycobacteria. Based on the essentiality of TopoI for the viability of *M. tuberculosis*, MtbTopoI is deemed as a validated target for discovery of novel TB drugs (Ravishankar et al., 2015; Nagaraja et al., 2017). Due to its involvement in transcription response to stress challenge, mycobacteria TopoI inhibitors could be especially useful in combination with other antibiotics.

Acknowledgments

The MsmTopoI truncated mutants (D1-D5, D1-D6, D1-D7, D1-D8) were constructed by Nan Cao. pKW08 plasmid with a MsmTopoI insert was constructed by Pamela Garcia Moreno.

CHAPTER 4

INVESTIGATING A DIRECT INTERACTION BETWEEN *ESCHERICHIA COLI* TOPOISOMERASE I AND RECA

ABSTRACT

Protein-protein interactions are of special importance in cellular processes, including replication, transcription, recombination, and repair. *Escherichia coli* topoisomerase I (EcTopoI) is primarily involved in the relaxation of negative DNA supercoiling. *E. coli* RecA, the key protein for homologous recombination and SOS DNA-damage response, has been shown to stimulate the relaxation activity of EcTopoI. The evidence for their direct protein-protein interaction has not been previously established. We report here the direct physical interaction between *E. coli* RecA and topoisomerase I. We demonstrated the RecA-topoisomerase I interaction via pull-down assays, and surface plasmon resonance measurements. Molecular docking supports the observation that the interaction involves the topoisomerase I N-terminal domains that form the active site. Our results from pull-down assays showed that ATP, although not required, enhances the RecA-EcTopoI interaction. We propose that *E. coli* RecA physically interacts with topoisomerase I to modulate the chromosomal DNA supercoiling.

INTRODUCTION

Protein-protein interactions (PPIs) are essential features of almost every cellular process (Coulombe et al., 2004; Perkins et al., 2010). Genomic processes including DNA replication, transcription, translation, recombination, and repair require an ensemble of

proteins (Coulombe et al., 2004). PPIs, especially transient protein interactions, are vital in the regulation of the above-mentioned genomic processes (Perkins et al., 2010; Ngounou Wetie et al., 2013). Proteins involved in transient interactions can function as independent units in the cells, and certain post-translational modifications on these proteins or binding of ligands can trigger the protein interactions. A protein's function is defined and controlled through interaction with other proteins, or biomolecules (Ngounou Wetie et al., 2013).

Understanding protein-protein interaction network in *Escherichia coli* would be essential in broadening current insight on the fundamental cellular processes. PPIs involved in DNA damage response would be important for the development of antibiotic resistance (Marceau et al., 2013). We are reporting here a direct physical interaction of RecA, the key player of homologous recombination and SOS DNA-damage response in *E. coli*, with DNA topoisomerase I. RecA family of recombinases, conserved in most of the bacteria, are ATP-dependent proteins mediating homologous recombination, DNA repair and genome integrity (Karlin and Brocchieri, 1996; Lin et al., 2006; Cox, 2007). Homolog searches have provided evidence for conservation of RecA in bacteria, archaea, and eukaryotes, although, the functions of the homologs have diversified with evolution. Most of the archaeal species have two RecA homologs (RadA and RadB), whereas the eukaryotes have multiple representatives of the RecA family (Rad51, Rad51B, Rad51C Rad51D, Dmc1, XRCC2, XRCC3, and RecA) (Lin et al., 2006). RecA monomers bind to single-stranded DNA (ssDNA) in an ATP-dependent manner forming an active nucleoprotein filament (McGrew and Knight, 2003; Bell, 2005). *E. coli* RecA, a prototype of RecA family of proteins, has multiple roles in the cell. RecA catalyzes the DNA strand exchange mechanism by coupling with ATP hydrolysis, promoting the recombination

process (Howard-Flanders et al., 1984; Cox, 1999; Cox, 2002; Lusetti and Cox, 2002; Cox, 2003). RecA can also function as a coprotease of LexA, and UmuD proteins. RecA facilitates the autocatalytic cleavage of LexA repressor, which is required for inducing the SOS response (Little, 1991; Harmon et al., 1996). It can also facilitate the autocatalytic cleavage of UmuD to an active UmuD', which is a component of a low fidelity DNA polymerase V that is involved in the translesion DNA synthesis (Patel et al., 2010).

The topology of DNA is maintained by an important group of evolutionarily conserved enzymes called topoisomerases (Wang, 2002). The essential genomic processes such as replication, transcription, recombination, and repair can create topological strain or entanglement on the double helix of DNA (Vos et al., 2011). Topoisomerases transiently cleave and rejoin DNA (Wang, 1971) to resolve the topological strain or entanglement, and maintain the genomic stability (reviewed in (Wang, 1971; Berger, 1998; Champoux, 2001; Chen et al., 2013)).

E. coli DNA topoisomerase I is primarily involved in the relaxation of negatively supercoiled DNA by the stand passage mechanism (Brown and Cozzarelli, 1981; Tse-Dinh, 1986; Champoux, 2002b). It has an important function in preventing excess negative supercoiling of DNA (Drlica, 1992) which can affect global transcription and result in growth inhibition. According to a previous report, the relaxation activity of *E. coli* topoisomerase I is stimulated by RecA; suggesting a functional interaction between RecA and topoisomerase I (Reckinger et al., 2007). It remains unclear whether this stimulatory effect is due to a direct protein-protein interaction between *E. coli* RecA and topoisomerase I, or is only due to the effect of *E. coli* RecA on DNA conformation. More recent results

showed that mutations in *E. coli topA* gene coding for topoisomerase I can diminish the *E. coli* SOS response to DNA damage and antibiotics treatment (Yang et al., 2015).

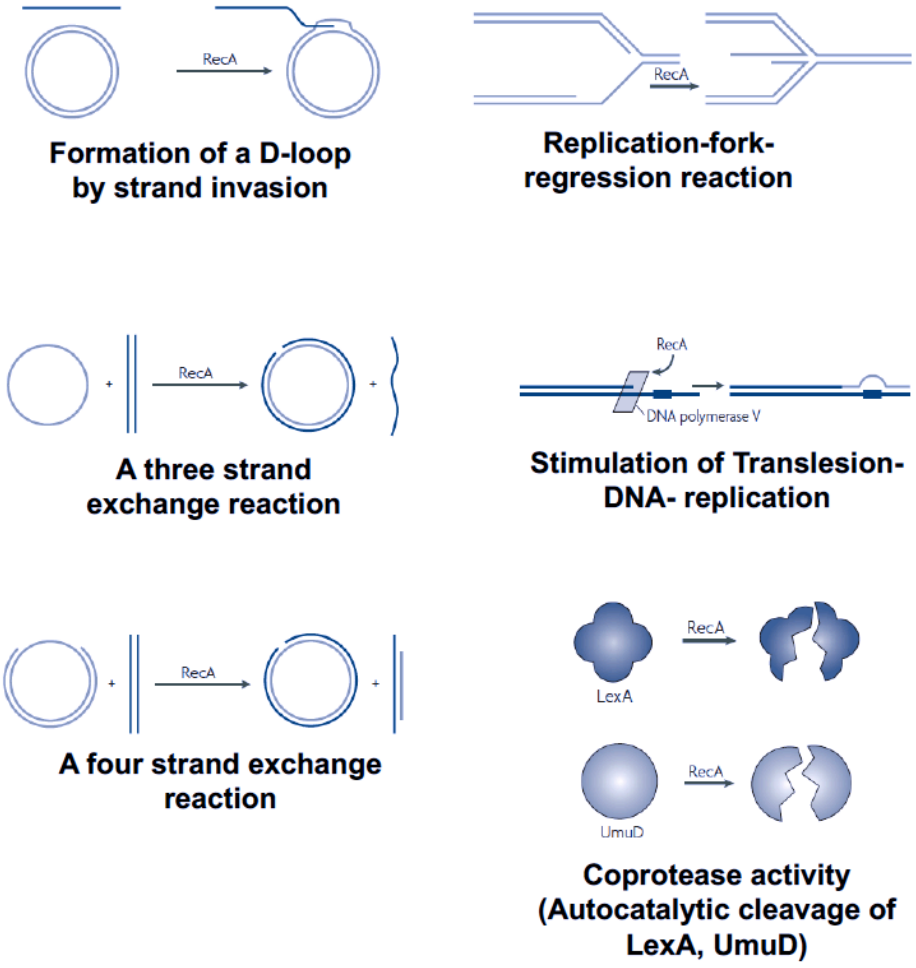


Figure 4. 1. Multiple activities of *E. coli* RecA (Cox, 2007)

The interaction between RecA and topoisomerase I may influence the increase in antibiotic resistance (Hastings et al., 2004; Beaber et al., 2004; Thi et al., 2011) and persistence shown to be associated with the SOS response (Dörr et al., 2009). In this study,

we tested the hypothesis that *E. coli* RecA might physically interact with topoisomerase I to modulate the topoisomerase I catalytic activity and DNA supercoiling.

Herein, we present evidence for a direct physical interaction between *E. coli* RecA, and topoisomerase I in solution by pull-down assays (Yang et al., 2015) as well assess the influence of ATP, and the domains of topoisomerase I involved in the protein-protein interaction with RecA. We further investigated the inter-protein interaction between *E. coli* RecA and topoisomerase I by using surface plasmon resonance (SPR) and molecular docking. SPR is a widely accepted label-free biophysical tool in order to investigate biomolecular interactions (Wilson, 2002; Willander & Al-Hilli, 2009; Tiwari et al., 2014), including PPIs (Berggård et al., 2007; Tiwari et al., 2015), whereas molecular docking can be used to provide structural insights for PPIs (Smith and Sternberg, 2002; Gray et al., 2003). The structural basis for the protein-protein interaction was predicted by molecular docking that shows the N-terminal domains (NTD) of topoisomerase I are involved in the interaction with RecA. The NTD (D1-D4, amino acids 1-597) contain the active site for DNA cleavage-religation (Lima et al., 1994). Experimental evidence supporting this prediction was provided from pull-down assays.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli strain BW25113 (*Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ⁻*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*), obtained from Yale CGSC (Datsenko and Wanner, 2000), was used for preparing the cell lysate used in the pull-down of RecA from total cellular proteins. Plasmid, pLIC-ETOP was used for the expression and purification of

recombinant *E. coli* topoisomerase I with 6x-His tag (Sorokin et al., 2008). A plasmid, pLIC-NTD-ETOP was constructed similarly as pLIC-ETOP by introducing the coding sequence of NTD of *E. coli* topoisomerase I (amino acids 1-597) into a pLIC-*HK* cloning vector that allows T7 RNA polymerase-dependent expression of His-tagged NTD of topoisomerase I for purification (Sorokin et al., 2008). A pET His_6-Mocr TEV cloning vector (2O-T) (gift of Scott Gradia, Addgene #29710) was used for expression and purification of a recombinant viral protein, His-Mocr (DelProposto et al., 2009), that was used as negative control in the pull-down assays.

Purified Proteins

E. coli topoisomerase I with a N-terminus 6x-Histidine tag (His-EcTopoI) was expressed from pLIC-ETOP in *E. coli* BL21-AI by induction with 1 mM IPTG, 0.02% L-Arabinose as described previously (Sorokin et al., 2008). N-terminal domains (D1-D4) of the *E. coli* topoisomerase I with a N-terminus 6x-Histidine tag (His-NTD-EcTopoI) was expressed from pLIC-NTD-ETOP in BL21 Star (DE3) by induction with 1 mM IPTG. Expression of recombinant His-tagged Mocr was induced in BL21 star (DE3) with 1 mM IPTG. Ni- Sepharose 6 Fast Flow beads (GE Healthcare Life Sciences) were used to purify these proteins by affinity chromatography (Cheung et al., 2012) to near homogeneity as described previously (Sorokin et al., 2008) with some modifications. Purified *E. coli* RecA was purchased from New England BioLab for use in assays involving verification of direct protein-protein interactions with topoisomerase I.

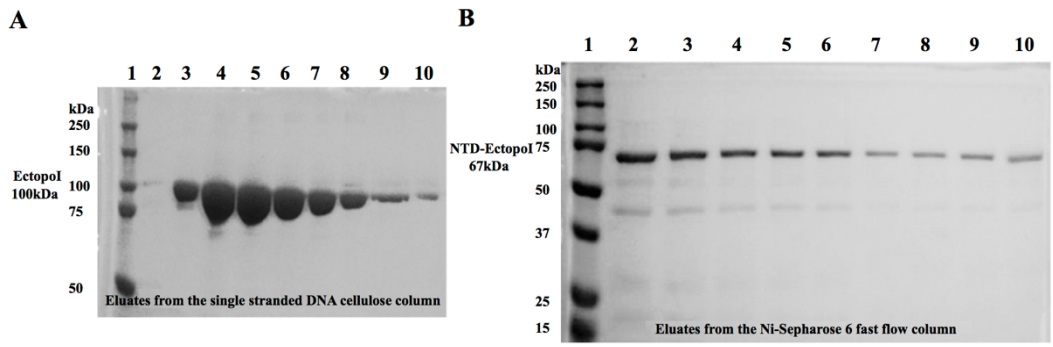


Figure 4.2. Purification of His-tagged *E. coli* topoisomerase I and its NTD:
(A) Purification of EcTopoI, Lane1: Protein color ladder, Lane 2-10: Eluates from the single stranded DNA cellulose column. (B) Purification of NTD-EcTopoI, Lane 1: Protein color ladder, Lane 2-10: Eluates from the Ni-Sepharose 6 fast flow column.

Recombinant EcTopoI with N-terminus 6xHis tag (His-EcTopoI) was expressed from exponential phase BL21-AI competent cells by induction with 1 mM IPTG, and 0.02% L-Arabinose. The soluble protein fraction obtained from lysing the cells was incubated with Ni-NTA agarose beads (Qiagen), and packed into a column after a few washes with PBS buffer (50 mM sodium phosphate, 300 mM sodium chloride; pH 8.0) containing 10 mM imidazole. To achieve maximum purity, further washes were carried out with PBS buffer containing 20mM Imidazole for at least 100 column volumes. The recombinant 6x-Histidine tagged EcTopoI was then eluted with 250mM Imidazole. The eluted recombinant protein was dialyzed to remove Imidazole, and passed through a single stranded DNA cellulose column, as described previously (Zhu & Tse-Dinh, 1999) (Figure 4.2, A). The recombinant NTD-topoisomerase I (NTD EcTOP1) was expressed from BL21 Star (DE3) cells by induction with 1mM IPTG at exponential growth phase. The purification protocol followed was similar to the protocol for recombinant EcTopoI purification, except here the soluble protein fraction was passed through a single column: Ni- sepharose 6 fast flow column (GE Healthcare) (Figure 4.2, B).

Pull-down assays to study direct physical interactions between purified proteins

Pull-down assays were carried out to establish the physical interactions of proteins in solution (Yang et al., 2015). An assay involving the incubation of purified RecA and topoisomerase I was carried out to study the direct physical interactions between these proteins. Purified His-EcTopoI serves as bait in these assays. Individual pull-down reactions were set up by incubating constant amount of bait (10 nM) with varying concentrations (0-80 nM) of RecA (prey) for 2 hours at 4°C. The bait-prey interactions were set up in pull-down buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 0.5 mM $MgCl_2$, 0.005% v/v Tween-20). The HisPur Cobalt Agarose resin (Thermofisher), previously equilibrated in the above-mentioned pull-down buffer, was mixed with the bait-prey reaction. Following an overnight incubation at 4°C, the reactions were centrifuged and the supernatant was discarded. The resin pellet was then washed three times in HEPES buffer, and the proteins bound to the resin were eluted with pull-down buffer containing 400 mM imidazole. The eluates were electrophoresed in a polyacrylamide SDS gel, and RecA was detected by western blotting (Burnette, 1981) with Anti-RecA monoclonal antibody (MBL International Corp.). A C-DiGit blot scanner (LI-COR) was used to detect the chemiluminescent western blot signal, and the signal intensity was quantified (Image Studio Digits version 4.0).

A comparative study was performed to compare the RecA-topoisomerase I binding efficiency in the presence, and absence of 5 mM ATP. The assay was carried out with a constant amount (10 nM) of His-EcTopoI as bait, and varying concentrations (0-80 nM) of RecA as prey. An independent similar assay was carried out with a constant amount (10

nM) of NTD-EcTopoI as bait, and varying RecA concentrations (0-60 nM) as prey in the presence of ATP.

Pull-down assays on *E. coli* soluble cell lysate

In this assay, *E. coli* strain (BW25113) cultured in LB medium for 16 hours to stationary phase (OD_{600}=2.5), and the culture was pelleted. The cell pellet was suspended in the pull-down buffer containing 1mg/ml lysozyme. The suspended cells were subjected to lysis by four freeze-thaw cycles. The lysate was centrifuged at 13000xg for 2 hours at 4°C. The soluble protein fraction was incubated with HisPur Cobalt Agarose resin (precleared) before its use in the pull-down reaction. The preclearing minimizes non-specific binding of proteins to the beads. Either full length purified EcTopoI or NTD-EcTopoI was used as bait. A Bacteriophage T7 protein, Mocr, with a N-terminus 6x-His tag was used as bait in the negative control for the pull-down assay (DelProposto et al., 2009).

Bait (40 nM), and precleared soluble protein fraction (150 µg) were incubated at 4°C for 2 hours. HisPur Cobalt Agarose resin (Thermofisher) was added to the reaction, and incubated overnight at 4°C. On the following day, the resin-reaction mixture was spun, and the supernatant was discarded. The bead pellet was washed three times in pull-down buffer with 10 mM imidazole to minimize non-specific binding of histidine rich proteins to the resin. The proteins bound to the resin were finally eluted in 400 mM imidazole, and the eluates were subjected to SDS-PAGE analysis. A western blot was performed to probe for RNA polymerase, and RecA in the eluates using a monoclonal antibody against RNA polymerase beta (BioLegend), and RecA respectively.

Surface Plasmon Resonance (SPR)

Biacore T200 SPR instrument was used to record SPR sensorgrams. EcTopoI was immobilized onto CM5 sensor surface using standard amine coupling chemistry. Buffered solutions with various concentrations of RecA were flown over EcTopoI immobilized sensor surface. EcTopoI in 10 mM sodium acetate buffer (pH 5.5) was immobilized onto a CM5 chip surface via standard amine-coupling chemistry. 1XHBS-P+ (10 M HEPES, pH 7.4, 150 mM NaCl, 0.05% surfactant P-20) was used as the immobilization running buffer. The remaining surface reactive ester groups were quenched using 1M Ethanolamine-HCl for 720 seconds. Buffered solutions of RecA protein (10 mM Tris-HCl; pH 7.5, 0.1 mM EDTA, 1 mM DTT, 10 mM MgCl2, 0.005% Tween-20 and 5% glycerol) at various concentrations were injected over EcTopoI saturated surface in the presence of RecA buffer. 1M NaCl was used to regenerate the sensor surface. The flow rate was maintained at 50 µL/min during the binding experiments and the data was collected at the rate of 10 Hz. All the experiments were carried out at 25^{o}C.

Molecular docking

The formation of inter-protein complex between EcTopoI and RecA was optimized using pyDockWEB (Jiménez-García et al., 2013). Protein coordinates from PDB entries 4RUL (full length EcTopoI, (Tan et al., 2015)) and 2REB (*E. coli* RecA, (Story et al., 1992)) were used in the docking study as receptor (EcTopoI) and ligand (RecA), respectively. The top ten docked complexes from the pyDockWEB outputs, based on energy scoring, were used to predict the RecA interaction site on EcTopoI. The output PDB file of the top scored complex was analyzed using PDBsum database (Laskowski et al.,

1997; Laskowski, 2001). Chimera molecular graphics software (Pettersen et al., 2004) was used to visualize the structure and to generate images of the docked complexes.

RESULTS

Pull-down assay demonstrates a direct physical interaction between *E. coli* RecA and topoisomerase I

A functional association between *E. coli* RecA and topoisomerase I have been reported previously (Cunningham et al. 1981; Reckinger et al., 2007). More recently, a role of topoisomerase I was observed in *E. coli* SOS response (Liu et al., 2011; Yang et al., 2015), which prompted us to verify the possibility of a direct physical interaction between these proteins. Purified His-EcTopoI and RecA were incubated together in the presence of ATP, and pulled-down with Cobalt agarose resin. The amount of RecA bound to EcTopoI was determined by western blot analysis of the eluates from the reaction with monoclonal antibodies against RecA. The results (Fig. 4.3) confirmed the possibility of a direct interaction between these proteins. Pull-down of RecA by the resin required the presence of His-EcTopoI. Both *E. coli* RecA, and topoisomerase I bind strongly to single-stranded DNA. However, according to this pull-down result with purified RecA and topoisomerase I, the association between these proteins does not require the presence of DNA.

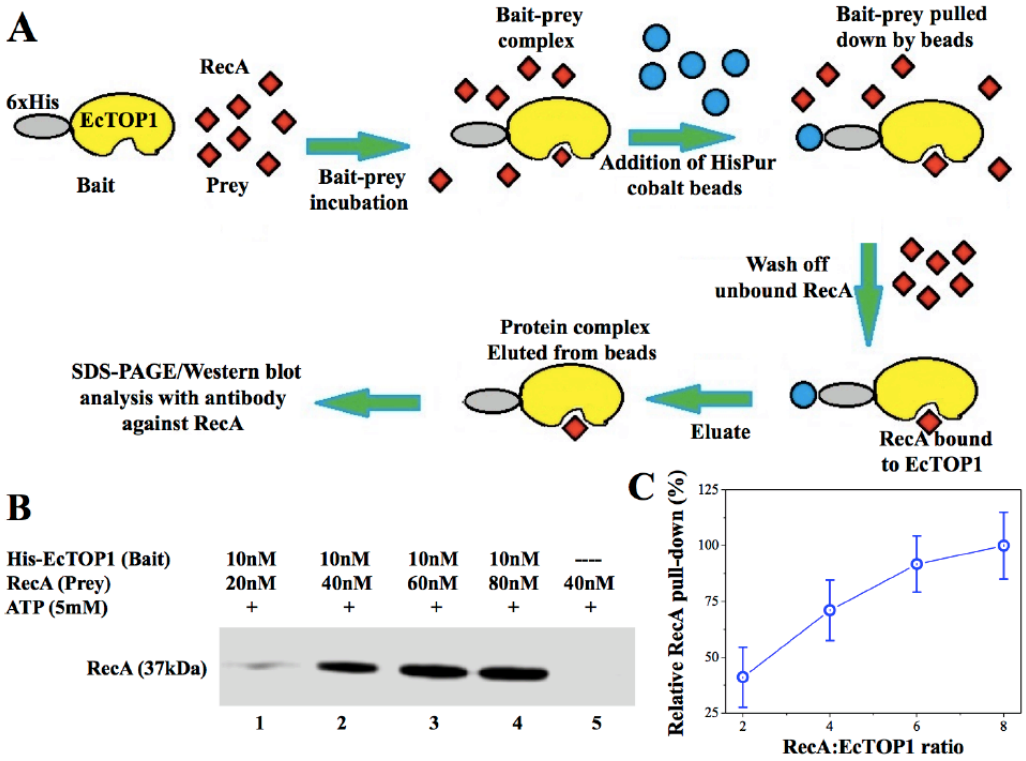


Figure 4.3. Direct physical interaction between purified *E. coli* RecA and topoisomerase I (A) Pull-down scheme (B) Pull-down of *E. coli* RecA by topoisomerase I at an increasing RecA: EcTopoI molar ratios, as measured by western blot using antibodies against RecA. Lanes 1-4: Eluates from pull-down reactions with increasing RecA: EcTopoI molar ratios. Lane 5: Negative control in the absence of EcTopoI. (C) Graph showing average values (symbols) of RecA band intensities, from three independent experiments, relative to the maximal intensity of RecA in the pull-down reactions. The error bars represent standard deviations of three measurements.

Influence of ATP on the binding efficiency of RecA with EcTopoI

The functional interactions between *E. coli* RecA and topoisomerase I were observed in the presence of ATP. According to a previous report (Konola et al., 1994), ATP binds to the P-loop of RecA. *E. coli* RecA undergoes ATP dependent conformational change (Cox, 2003) that could affect its interaction with topoisomerase I (Cunningham et al. 1981; Reckinger et al., 2007). We, therefore tested the influence of ATP on the physical

interaction between *E. coli* RecA and topoisomerase I with pull-down reactions in the absence or presence of ATP.

While the results from the pull-down assay suggested that the protein-protein interaction between *E. coli* RecA and topoisomerase I may not require ATP, the presence of ATP in the pull-down assay was found to enhance the protein-protein interaction significantly (Fig.4.4, A; 4.4, B). Experimental data from one trial of pull-down experiment is shown here (Fig.4.4, A). Similar enhancement of the interaction by the presence of ATP were seen in two additional trials of the experiment (Fig. 4.4, C). However, ATP did not appear to be absolutely required for the interaction. Direct protein interaction between *E. coli* RecA and topoisomerase I in the absence of ATP has been confirmed by surface plasmon resonance (SPR) measurements (Fig. 4.4, D; 4.4, E). We could not obtain meaningful SPR sensorgrams for RecA-EcTopoI interactions in the presence of ATP due to technical difficulties. Fig. 4.4, D depicts the SPR sensorgrams for RecA binding to EcTopoI at various RecA (analyte) concentrations. The sensorgrams for each concentration represent the triplets. The SPR sensorgrams were evaluated using Biaevaluation software version 2.0 via 1:1 kinetics binding analysis (Wang et al., 2012). Since the signals for RecA-EcTOP1 complex dissociation were relatively slower, the dissociation rate constant (k_d) value was determined by fitting the highest analyte concentration data (Fig. 4.4, E), with dissociation signals recorded for longer time as compared to lower concentrations. The association rate constant (k_a) was determined by fitting the full set of sensorgrams. The equilibrium dissociation constant ($K_D=k_d/k_a$), for the direct binding of RecA with EcTopoI was determined to be ~1500 nM in the absence of ATP. The RecA-EcTOP1 binding experiments were also conducted in the presence of

ATP in the RecA solution (analyte mixture). However, the strong binding of the analyte mixture in the reference flow cell hindered meaningful SPR sensorgrams.

Molecular docking results for the complex formation between RecA and EcTopoI

Fig. 4.5 depicts the binding complex, as predicted by pyDockWEB, between EcTopoI (receptor) and RecA (ligand). Fig. 4.5, A shows the surface representation for the binding of EcTopoI (green) and with RecA (10 different colors, except green, representing the RecA conformations upon binding with EcTopoI). Fig. 4.5, B shows the cartoon representation for the top-scored EcTopoI-RecA docked complex as well as the interacting amino acid residues, as predicted by PDBsum, across the binding interface. The amino acid residues predicted to be responsible for the formation of hydrogen bonds and salt bridges are listed in the tables 4.T1, 4.T2.

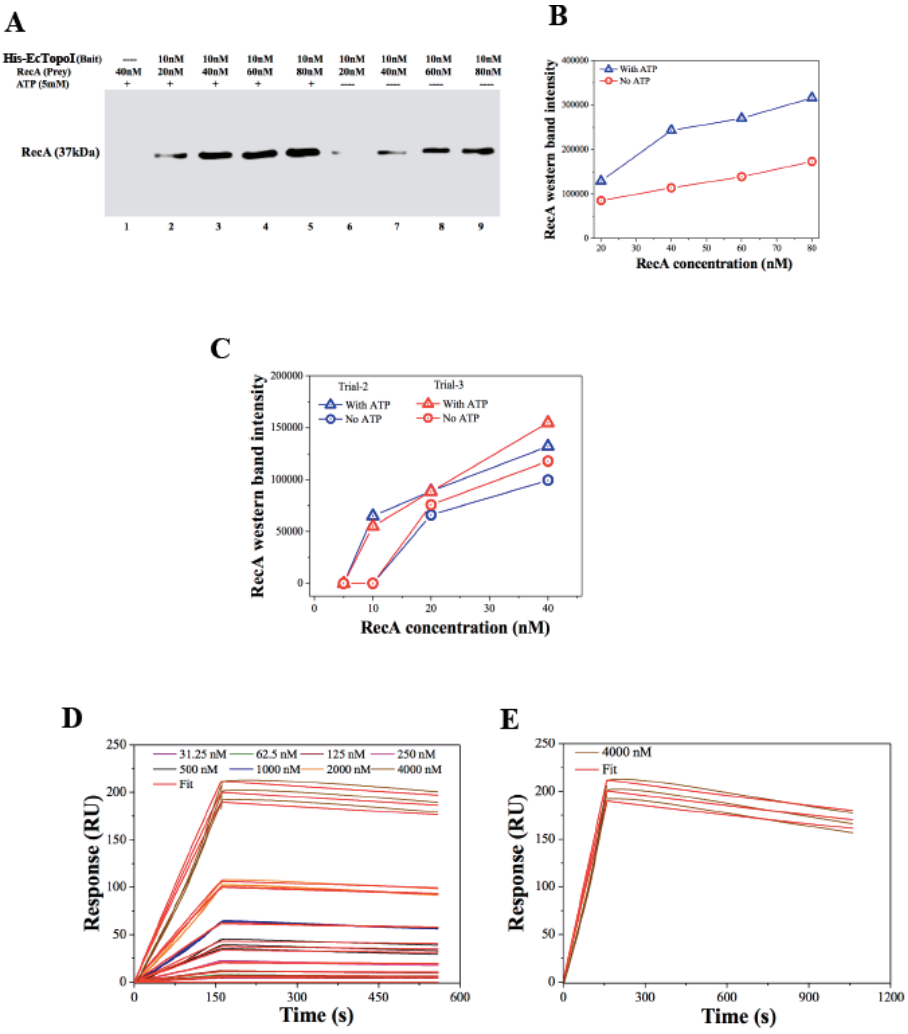


Fig. 4.4. ATP promotes binding of *E. coli* RecA to topoisomerase I. (A) Comparative analysis of ATP's influence on the direct protein interactions between RecA, and EcTopoI. Lanes 2-5: Eluates from pull-down reactions in the presence of 5 mM ATP. Lanes 6-9: Eluates of pull down reactions devoid of ATP. Lane 1: negative control (No EcTopoI (bait) in the reaction). (B) The quantified RecA band intensities. (C) Results from repeated trials of pull-down assays showing the influence of ATP on RecA-EcTopoI complex formation. (D) SPR sensorgrams for RecA binding to immobilized EcTopoI (E) SPR sensorgrams for RecA binding binding at 4000 nM RecA concentration with dissociation signals recorded for a longer time as compared to lower RecA concentrations. The sensorgrams for each concentration were recorded in triplets. The SPR sensorgrams were fitted to 1:1 kinetics binding model using Biaevaluation software version 2.0. The red colored lines are the fits.

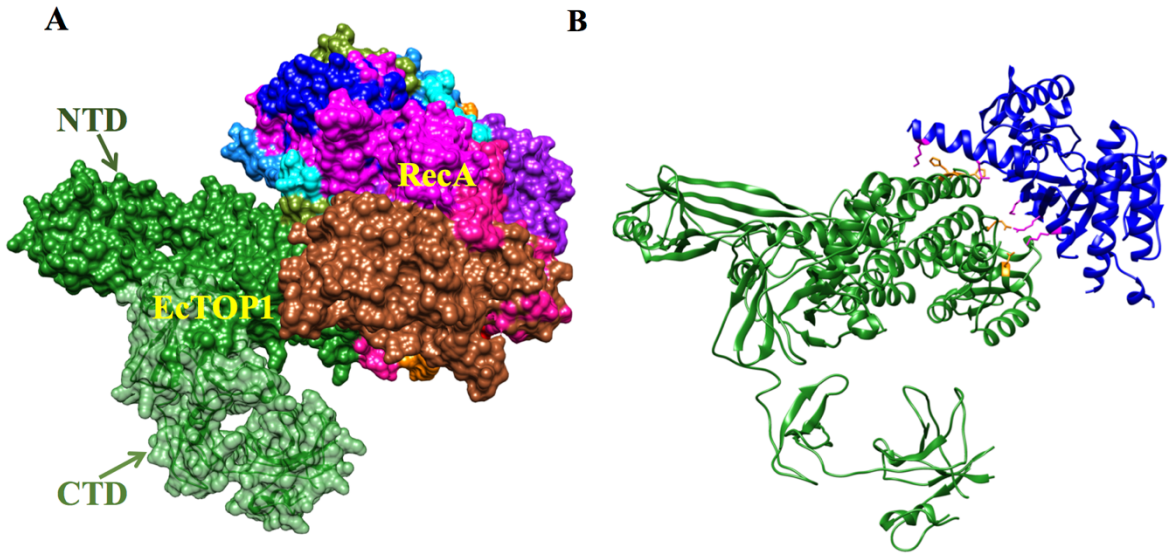


Figure 4.5. RecA-EcTopoI complex predicted by molecular docking: (A) Green colored surface represents EcTopoI with light green as its C-terminal domain (CTD) and dark green as N-terminal domain (NTD). The surfaces in the other colors represent ten different predicted RecA conformations when it binds to EcTopoI, all with NTD of EcTopoI as the binding domain interacting with RecA. (B) Cartoon representation of the top scored docked RecA- EcTopoI complex. EcTopoI is shown in green color and RecA in blue color. The amino acid residues across the EcTopoI-RecA binding interface that form hydrogen bonds and salt bridges are shown in sticks representation (orange colored sticks for EcTopoI and magenta colored sticks for RecA).

Amino acid residues predicted to be involved in the formation of hydrogen bonds and salt bridges:

The potential amino acid residues for the formation of hydrogen bonds and salt bridges, as predicted by PDBsum, across the binding interface of EcTopoI-RecA complex are listed in Table 4. T1 and 4. T2, respectively.

Table 4. T1. Potential amino acid residues for the formation of hydrogen bonds during EcTopoI-RecA complex formation.

EcTopoI	RecA
GLU (156)	ARG (226)
HIS (556)	LEU (29)
HIS (566)	LYS (6)

Table 4. T2. Potential amino acid residues for the formation of salt bridges during EcTopoI-RecA complex formation.

EcTopoI	RecA
GLU (125)	LYS (216)
GLU (156)	ARG (226)
GLU (156)	LYS (245)

Pull-down assay for complex formation between NTD-EcTopoI and RecA

Molecular docking results have suggested that the NTD of EcTopoI can interact with RecA in *E. coli*. The possibility of the direct interaction of RecA with the NTD-EcTopoI was verified by pull-down assays, involving the direct incubation of purified recombinant NTD-EcTopoI and RecA, in the presence of ATP. In these assays, NTD-EcTopoI (bait) and RecA (prey) were incubated with HisPur Cobalt agarose resin, in the presence of ATP. The eluates from the pull-down reactions were analyzed by western blotting with monoclonal RecA antibodies. The results from the assay suggest that the N-terminal domain of topoisomerase I and RecA can interact physically (Fig. 4.6).

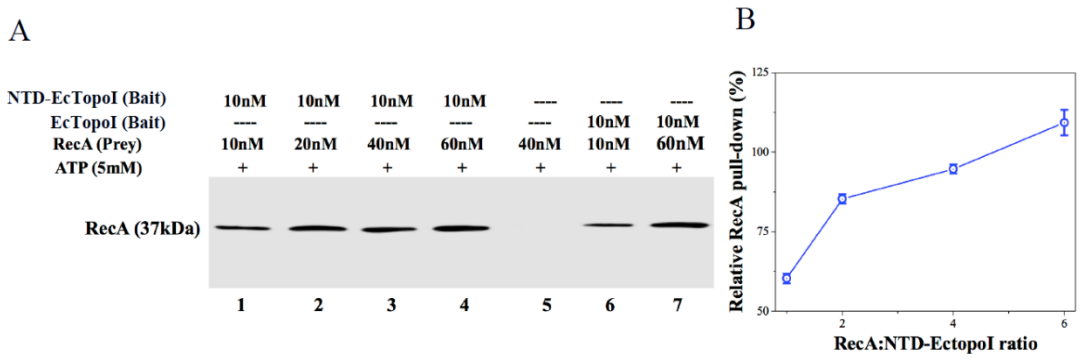


Figure 4.6. NTD of EcTopoI can interact with RecA as efficiently as full-length EcTopoI. (A) Pull-down of RecA, at an increasing RecA: NTD-EcTopoI molar ratios, as measured by western blot using antibodies against RecA. Lane 1-4: Eluates from the pull-down reactions of increasing RecA (prey) to NTD-EcTopoI (bait) in the presence of ATP. Lane 5: negative control for the assay (The reaction has beads incubated with prey, in the absence of bait). Lane 6, 7: Eluates from reaction carried out with full-length EcTopoI, as bait. (B) Quantified RecA band intensities relative to the band intensity observed with pull-down reaction corresponding to 1: 6 molar ratios of EcTopoI and RecA in lane 7 of Fig. 4.6, A. The average values of three trials are shown here and the error bars represent the standard deviations.

Pull-down of RecA from E. coli soluble cell lysate by recombinant EcTopoI and NTD-EcTopoI

E. coli RecA has a stimulatory effect on the topoisomerase I relaxation activity, suggesting a possible protein-protein interaction between RecA and topoisomerase I (Reckinger et al., 2007). A direct physical interaction between RecA and EcTopoI with purified proteins in solution was verified (Fig 4.3). A pull-down assay using the *E. coli* cell lysate was performed to further confirm the interaction of *E. coli* topoisomerase I-NTD with *E. coli* RecA. The cell lysate, and His-EcTopoI or His-NTD-EcTopoI were incubated together with HisPur Cobalt agarose beads that have high affinity for the 6x-Histidine tag. The complexes recovered from the beads after the pull-down protocol were resolved by SDS-PAGE, and analyzed by western blot. The nitrocellulose membrane was probed for RecA and RNA polymerase with monoclonal antibodies against RecA and RNA polymerase beta subunit respectively.

EcTopoI is known to interact with *E. coli* RNA polymerase via its CTD (Cheng et al., 2003). The results showed that both RecA, and RNA polymerase were pulled down by full-length topoisomerase I as expected (Fig. 4.7, lane 2). The data also confirmed that the NTD of topoisomerase I can interact with RecA in the cell lysate (Fig. 4.7, lane 3). Interaction between NTD-EcTopoI and RNA polymerase was not observed, demonstrating the domain specific interactions between EcTopoI and its partners (Cheng et al., 2003).

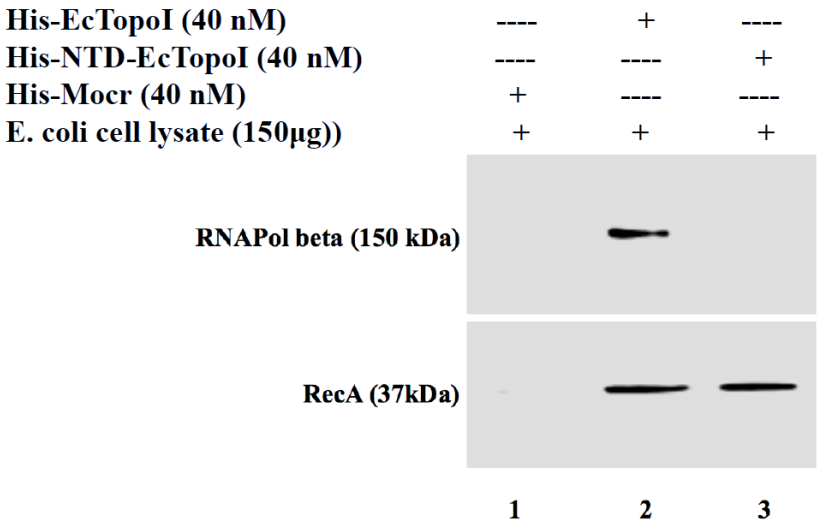


Fig. 4.7 Pull-down of RecA from *E. coli* cell lysates by EcTopoI and NTD-EcTopoI. Lane 2 and 3 represent the eluates from the pull-down reactions containing His-EcTopoI, His-NTD-EcTopoI as bait respectively. Lane 1, representing the eluate from the pull-down reaction with Mocr as bait, serves as a negative control.

DISCUSSION

In a previous study (Reckinger et al., 2007), the stimulation of topoisomerase I relaxation activity by *E. coli* RecA was seen only for topoisomerase I protein from *E. coli* and not for the topoisomerase I proteins from other species. This indicated that the stimulation of relaxation activity by RecA was not entirely due to the effect of RecA on DNA conformation. Even though, previous studies suggested that *E. coli* RecA may stimulate topoisomerase I relaxation activity via direct protein-protein interaction, data to support such interaction was not available (Reckinger et al., 2007). We have presented evidence, here, for the first time to confirm a direct physical interaction between *E. coli* RecA and topoisomerase I. The presence of DNA was not required for this interaction. ATP, although not absolutely required, can enhance the protein-protein interaction between

E. coli RecA and topoisomerase I. *E. coli* topoisomerase I plays an important role in the regulation of local and global DNA supercoiling (Liu and Wang, 1987; Drlica, 1992). The stimulation of topoisomerase I relaxation activity by RecA via direct protein-protein interaction allows RecA to add modulation of DNA supercoiling to its multiple roles. This stimulation of topoisomerase I relaxation activity by RecA may enable relaxation-dependent *E. coli* promoters to have higher transcription activities following DNA damage (Reckinger et al., 2007).

As a type IA DNA topoisomerase, EcTopoI binds to the single-stranded region of negatively supercoiled DNA to initiate its relaxation activity (Champoux, 2001). The active site of EcTopoI (Tyr-319 nucleophile), responsible for single-stranded DNA cleavage and religation, is located in its NTD. The active site region formed an interface between the subdomains that enclose the toroid hole in its structure (Lima et al., 1994; Berger, 1998). Molecular docking and pull-down results reported here has provided evidence for a physical interaction of RecA with the NTD of topoisomerase I. The protein-protein interactions may either facilitate the loading of negatively supercoiled DNA onto topoisomerase I, or increase the catalytic rate of DNA relaxation by inducing conformational change in topoisomerase I. It is notable that EcTopoI interacts with RNA polymerase via its CTD (Cheng et al., 2003), so that the transcription-driven negative supercoiling can be relaxed efficiently to prevent hyper-negative supercoiling of DNA and suppress R-loop stabilization (Tan et al., 2015). Interaction with RecA takes place via a different domain in topoisomerase I and may also have functional significance in the physiological response of *E. coli* to DNA damage and antibiotic stress. Future studies will

further investigate the mechanism of the RecA-topoisomerase I interaction, and the physiological consequence of perturbation of this specific protein-protein interaction.

Acknowledgments

Molecular docking, simulation studies and SPR experiments were carried out by Purushottam Babu Tiwari (Georgetown University).

SUMMARY AND FUTURE WORK

Communication between proteins is vital for essential cellular processes like transcription, replication, recombination and DNA repair. Understanding the network of a protein can provide insights into its functions in a physiological setting. The goal of my work is to identify novel protein partners of bacterial topoisomerase I, and explore the molecular mechanisms for interaction. Bacterial topoisomerase I, a type IA topoisomerase, is involved in resolving the hyper negative supercoiling of DNA in bacteria. The physical and functional association of the CTD of topoisomerase I with RNAP beta' subunit in *E. coli* was previously reported. However, the molecular mechanism of interaction between these proteins was unknown. In chapter 1, the molecular mechanism of interaction between *E. coli* RNA polymerase and topoisomerase I was explored, and the findings were reported. The computation modeling (molecular docking, MD simulations) and pull-down experiments have identified key amino residues of EcTopoI involved in the salt-bridging and hydrogen bonding with RNAP. According to these studies, Lysine 664 and 627 of EcTopoI are essential for the formation and stabilization of the complex. It will be interesting to study the in vivo effects of mutating the Lysine residues (627, 664) of

EcTopoI. Currently, a mutant *E. coli* strain is under construction in Dr. Marc Drolet's lab (University of Montreal).

In chapter 2, novel protein partners of mycobacterial topoisomerase I were explored. Pull-down and Co-Immunoprecipitation assays (Co-IP) coupled to mass spectrometry were used to identify the protein network of mycobacterial topoisomerase I, MsmTopoI. RNAP and Putative helicases were identified as partners of MsmTopoI. The putative helicase will be characterized, in the future, for its helicase activity. According to the preliminary data, the putative DEAD/DEAH Box helicase is a partial 3'-5' DNA helicase. However, its RNA helicase activity should be verified. The mechanism of RNAP-topoisomerase I interaction in mycobacteria was investigated and reported in chapter 3. Unlike in *E. coli*, a novel mechanism of interaction with RNAP beta' subunit through the C-terminal tail of mycobacterial topoisomerase I was identified. The physiological relevance of the RNAP-topoisomerase I interaction during antibiotic and oxidative stress was also reported.

The evidence for a direct physical interaction between the N-terminal domain of topoisomerase I, and RecA was reported in chapter 4. The physical interaction does not require DNA or ATP, although it was observed that ATP can improve the interaction efficiency. In the future, it will be essential to study the molecular mechanisms of RecA-topoisomerase I interaction in *E. coli*. In addition, the cellular response of perturbing RecA-topoisomerase I association should be studied.

REFERENCES

Addo, K., Owusu-Darko, K., Yeboah-Manu, D., Caulley, P., Minamikawa, M., Bonsu, F., . . . Ofori-Adjei, D. (2007). Mycobacterial species causing pulmonary tuberculosis at the korle bu teaching hospital, accra, ghana. *Ghana Medical Journal, 41*(2), 52-57.

Aedo, S., & Tse-Dinh, Y. (2012). Isolation and quantitation of topoisomerase complexes accumulated on *Escherichia coli* chromosomal DNA. *Antimicrobial Agents and Chemotherapy, 56*(11), 5458-5464.

Aggarwal, M., & Brosh, R. M.,Jr. (2009). Premature aging syndrome gene WRN genetically interacts with a topoisomerase. *Cell Cycle (Georgetown, Tex.), 8*(14), 2143.

Ahmed, W., Menon, S., Godbole, A. A., Karthik, P. V., & Nagaraja, V. (2014). Conditional silencing of topoisomerase I gene of mycobacterium tuberculosis validates its essentiality for cell survival. *FEMS Microbiology Letters, 353*(2), 116-123.

Ahmed, W., Bhat, A. G., Leelaram, M. N., Menon, S., & Nagaraja, V. (2013). Carboxyl terminal domain basic amino acids of mycobacterial topoisomerase I bind DNA to promote strand passage. *Nucleic Acids Research, 41*(15), 7462-7471.

Ahmed, W., Menon, S., Karthik, P. V., & Nagaraja, V. (2015). Reduction in DNA topoisomerase I level affects growth, phenotype and nucleoid architecture of mycobacterium smegmatis. *Microbiology, 161*(2), 341-353.

Aldred, K. J., Kerns, R. J., & Osheroff, N. (2014). Mechanism of quinolone action and resistance. *Biochemistry, 53*(10), 1565-1574.

Alessandro Vindigni, H. I. (2009). RecQ helicases: Multiple structures for multiple functions? *Hfsp,*3(3), 153-64.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research, 25*(17), 3389-3402.

Andrea Von Groll, Anandi Martin, Pontus Jureen, Sven Hoffner, Peter Vandamme, Fran¸coise Portaels, . . . Brazil. (2009). *Fluoroquinolone resistance in mycobacterium tuberculosis and mutations in gyrA and gyrB* American Society for Microbiology.

Annamalai, T., Dani, N., Cheng, B., & Tse-Dinh, Y. (2009). Analysis of DNA relaxation and cleavage activities of recombinant *Mycobacterium tuberculosis* DNA topoisomerase I from a new expression and purification protocol. *BMC Biochemistry, 10*, 18.

Andrusier, N., Nussinov, R., & Wolfson, H. J. (2007). FireDock: Fast interaction refinement in molecular docking. *Proteins, 69*(1), 139-159.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., . . . Mori, H. (2006). Construction of escherichia coli K-12 in-frame, single-gene knockout mutants: The keio collection. *Molecular Systems Biology, 2*, 2006.0008.

Bachrati, C. Z., & Hickson, I. D. (2008). RecQ helicases: Guardian angels of the DNA replication fork. *Chromosoma, 117*(3), 219-233. doi:10.1007/s00412-007-0142-4

Baker, N. M., Rajan, R., & Mondragón, A. (2009). Structural studies of type I topoisomerases. *Nucleic Acids Research, 37*(3), 693-701. doi:10.1093/nar/gkn1009

Banda, S., Tiwari, P. B., Darici, Y., & Tse-Dinh, Y. (2016). Investigating direct interaction between *Escherichia coli* topoisomerase I and RecA. *Gene, 585*(1), 65-70.

Bansal, S., Tawar, U., Singh, M., Nikravesh, A., Good, L., & Tandon, V. (2010). Old class but new dimethoxy analogue of benzimidazole: A bacterial topoisomerase I inhibitor. *International Journal of Antimicrobial Agents, 35*(2), 186-190.

Beaber, J. W., Hochhut, B., & Waldor, M. K. (2004). SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature, 427*(6969), 72-74.

Bell, C. E. (2005). Structure and mechanism of *Escherichia coli* RecA ATPase. *Molecular Microbiology, 58*(2), 358-366. doi: MMI4876 [pii]

Bell, C. E., & Lewis, M. (2000). A closer view of the conformation of the lac repressor bound to operator. *Nature Structural Biology, 7*(3), 209-214.

Belova, G. I., Prasad, R., Kozyavkin, S. A., Lake, J. A., Wilson, S. H., & Slesarev, A. I. (2001). A type IB topoisomerase with DNA repair activities. *Proceedings of the National Academy of Sciences, 98*(11), 6015-6020. doi:10.1073/pnas.111040498

Beran-Steed, R. K., & Tse-Dinh, Y. (1989). The carboxyl terminal domain of *Escherichia coli* DNA topoisomerase I confers higher affinity to DNA. *Proteins: Structure, Function, and Bioinformatics, 6*(3), 249-258. doi:10.1002/prot.340060307

Berger, J. M. (1998). Structure of DNA topoisomerases. *Biochimica Et Biophysica Acta (BBA) - Gene Structure and Expression, 1400*(1–3), 3-18. doi:10.1016/S0167-4781(98)00124-9

Bergendahl, V., Thompson, N. E., Foley, K. M., Olson, B. M., & Burgess, R. R. (2003). A cross-reactive polyol-responsive monoclonal antibody useful for isolation of core RNA polymerase from many bacterial species. *Protein Expression and Purification, 31*(1), 155-160.

Bergerat, A., de Massy, B., Gadelle, D., Varoutas, P. C., Nicolas, A., & Forterre, P. (1997). An atypical topoisomerase II from archaea with implications for meiotic recombination. *Nature, 386*(6623), 414-417. doi:10.1038/386414a0

Berggård, T., Linse, S., & James, P. (2007). Methods for the detection and analysis of protein-protein interactions. *Proteomics, 7*(16), 2833-2842. doi:10.1002/pmic.200700131

Bernstein, D. A., & Keck, J. L. (2005). Conferring substrate specificity to DNA helicases: Role of the RecQ HRDC domain. *Structure (London, England : 1993), 13*(8), 1173-1182. doi:S0969-2126(05)00201-7 [pii]

Bhaduri, T., Bagui, T. K., Sikder, D., & Nagaraja, V. (1998). DNA topoisomerase I from *Mycobacterium smegmatis*. An enzyme with distinct features. *The Journal of Biological Chemistry, 273*(22), 13925-13932.

Blower, T. R., Williamson, B. H., Kerns, R. J., & Berger, J. M. (2016). Crystal structure and stability of gyrase-fluoroquinolone cleaved complexes from *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America, 113*(7), 1706-1713. doi:10.1073/pnas.1525047113

Bohr, V. A. (2008). Rising from the RecQ-age: The role of human RecQ helicases in genome maintenance. *Trends in Biochemical Sciences, 33*(12), 609-620. doi:10.1016/j.tibs.2008.09.003 [doi]

Borowiec, J. A. (2004). The toposome: A new twist on topoisomerase IIalpha. *Cell Cycle (Georgetown, Tex.), 3*(5), 627-628. doi:10.4161/cc.3.5.822

Bouthier de la Tour, C, Portemer, C., Kaltoum, H., & Duguet, M. (1998). Reverse gyrase from the hyperthermophilic bacterium *Thermotoga maritima*: Properties and gene structure. *Journal of Bacteriology, 180*(2), 274-281.

Brosh, R. M.,Jr, & Bohr, V. A. (2007). Human premature aging, DNA repair and RecQ helicases. *Nucleic Acids Research, 35*(22), 7527-7544. doi:gkm1008 [pii]

Brown, P. O., & Cozzarelli, N. R. (1979). A sign inversion mechanism for enzymatic supercoiling of DNA. *Science (New York, N.Y.), 206*(4422), 1081-1083.

Brown, P. O., & Cozzarelli, N. R. (1981). Catenation and knotting of duplex DNA by type 1 topoisomerases: A mechanistic parallel with type 2 topoisomerases. *Proceedings of the National Academy of Sciences of the United States of America, 78*(2), 843-847.

Brymora, A., Valova, V. A., & Robinson, P. J. (2004). Protein-protein interactions identified by pull-down experiments and mass spectrometry. *Current Protocols in Cell Biology, Chapter 17*, Unit 17.5. doi:10.1002/0471143030.cb1705s22

Burnette, W. N. (1981). "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry, 112*(2), 195-203.

Bush, N. G., Evans-Roberts, K., & Maxwell, A. (2015). DNA topoisomerases. *EcoSal Plus, 6*(2) doi:10.1128/ecosalplus. ESP-0010-2014

Cabrita, L. D., Dai, W., & Bottomley, S. P. (2006). A family of *E. coli* expression vectors for laboratory scale and high throughput soluble protein production. *BMC Biotechnology, 6*, 12. doi:10.1186/1472-6750-6-12

Champoux, J. J. (2001). DNA topoisomerases: Structure, function, and mechanism. *Annual Review of Biochemistry, 70*, 369-413. doi:70/1/369 [pii]

Champoux, J. J. (2002a). A first view of the structure of a type IA topoisomerase with bound DNA. *Trends in Pharmacological Sciences, 23*(5), 199-201.

Champoux, J. J. (2002b). Type IA DNA topoisomerases: Strictly one step at a time. *Proceedings of the National Academy of Sciences of the United States of America, 99*(19), 11998-12000. doi:10.1073/pnas.202483499

Champoux, J. J., & Dulbecco, R. (1972). An activity from mammalian cells that untwists superhelical DNA—A possible swivel for DNA replication. *Proceedings of the National Academy of Sciences of the United States of America, 69*(1), 143-146. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC427563/

Chen, S. H., Chan, N. L., & Hsieh, T. S. (2013). New mechanistic and functional insights into DNA topoisomerases. *Annual Review of Biochemistry, 82*, 139-170. doi:10.1146/annurev-biochem-061809-100002 [doi]

Cheng, B., Zhu, C. X., Ji, C., Ahumada, A., & Tse-Dinh, Y. C. (2003). Direct interaction between *Escherichia coli* RNA polymerase and the zinc ribbon domains of DNA topoisomerase I. *The Journal of Biological Chemistry, 278*(33), 30705-30710. doi:10.1074/jbc.M303403200 [doi]

Cheng, B., Shukla, S., Vasunilashorn, S., Mukhopadhyay, S., & Tse-Dinh, Y. (2005). Bacterial cell killing mediated by topoisomerase I DNA cleavage activity. *The Journal of Biological Chemistry, 280*(46), 38489-38495.

Chang, M., Delgado-Cruzata, L., Desai, R., Boone, C., Brown, G. W., Zhang, C., . . . Bellaoui, M. (2005). RMI1/NCE4 , a suppressor of genome instability, encodes a member of the RecQ helicase/topo III complex. *The EMBO Journal, 24*(11), 2024-2033. doi:10.1038/sj.emboj.7600684

Cheng, B., Liu, I. -., & Tse-Dinh, Y. (2007). Compounds with antibacterial activity that enhance DNA cleavage by bacterial DNA topoisomerase I. *The Journal of Antimicrobial Chemotherapy, 59*(4), 640-645. doi:10.1093/jac/dkl556

Cheok, C. F., Wu, L., Garcia, P. L., Janscak, P., & Hickson, I. D. (2005). The bloom's syndrome helicase promotes the annealing of complementary single-stranded DNA. *Nucleic Acids Research, 33*(12), 3932-3941. doi:33/12/3932 [pii]

Cheung, R. C., Wong, J. H., & Ng, T. B. (2012). Immobilized metal ion affinity chromatography: A review on its applications. *Applied Microbiology and Biotechnology, 96*(6), 1411-1420. doi:10.1007/s00253-012-4507-0; 10.1007/s00253-012-4507-0

Coulombe, B., Jeronimo, C., Langelier, M., Cojocaru, M., & Bergeron, D. (2004). Interaction networks of the molecular machines that decode, replicate, and maintain the integrity of the human genome. *Molecular & Cellular Proteomics: MCP, 3*(9), 851-856. doi:10.1074/mcp.R400009-MCP200

Cooper, M. A. (2002). Optical biosensors in drug discovery. *Nature Reviews. Drug Discovery, 1*(7), 515-528. doi:10.1038/nrd838 [doi]

Corbett, A. H., & Osheroff, N. (1993). When good enzymes go bad: Conversion of topoisomerase II to a cellular toxin by antineoplastic drugs. *Chemical Research in Toxicology, 6*(5), 585-597.

Corbett, K. D., & Berger, J. M. (2004). Structure, molecular mechanisms, and evolutionary relationships in DNA topoisomerases. *Annual Review of Biophysics and Biomolecular Structure, 33*, 95-118.

Cordone, A., Audrain, B., Calabrese, I., Euphrasie, D., & Reyrat, J. M. (2011). Characterization of a *Mycobacterium smegmatis* uvrA mutant impaired in dormancy induced by hypoxia and low carbon concentration. *BMC Microbiology, 11*, 231. doi:10.1186/1471-2180-11-231 [doi]

Cox, M. M. (1999). Recombinational DNA repair in bacteria and the RecA protein. *Progress in Nucleic Acid Research and Molecular Biology, 63*, 311-366.

Cox, M. M. (2002). The nonmutagenic repair of broken replication forks via recombination. *Mutation Research, 510*(1-2), 107-120. doi:S0027510702002567 [pii]

Cox, M. M. (2003). The bacterial RecA protein as a motor protein. *Annual Review of Microbiology, 57*, 551-577. doi:10.1146/annurev.micro.57.030502.090953 [doi]

Cox, M. M. (2007). Motoring along with the bacterial RecA protein. *Nature Reviews. Molecular Cell Biology, 8*(2), 127-138. doi:nrm2099 [pii]

Cramer, P. (2002). Multisubunit RNA polymerases. *Current Opinion in Structural Biology, 12*(1), 89-97.

Cunningham, R. P., Wu, A. M., Shibata, T., DasGupta, C., & Radding, C. M. (1981). Homologous pairing and topological linkage of DNA molecules by combined action of *E. coli* RecA protein and topoisomerase I. *Cell, 24*(1), 213-223.

Czubaty, A., Girstun, A., Kowalska-Loth, B., Trzcińska, A. M., Purta, E., Winczura, A., . . . Staroń, K. (2005). Proteomic analysis of complexes formed by human topoisomerase I. *Biochimica Et Biophysica Acta, 1749*(1), 133-141. doi:10.1016/j.bbapap.2005.03.007

Darden, T., York, D., & Pedersen, L. (1993). Particle mesh ewald: An n·log(N) method for ewald sums in large systems. *The Journal of Chemical Physics, 98*(12), 10089-10092. doi:10.1063/1.464397

Dawson, R., & Diacon, A. (2013). PA-824, moxifloxacin and pyrazinamide combination therapy for tuberculosis. *Expert Opinion on Investigational Drugs, 22*(7), 927-932.

Datsenko, K. A., & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences of the United States of America, 97*(12), 6640-6645. doi:10.1073/pnas.120163297 [doi]

De Las Rivas, J., & Fontanillo, C. (2010). Protein-protein interactions essentials: Key concepts to building and analyzing interactome networks. *PLoS Computational Biology, 6*(6), e1000807. doi:10.1371/journal.pcbi.1000807

Déclais, A. C., Bouthier, C., & Duguet, M. (2001). Reverse gyrases from bacteria and archaea. *Methods in Enzymology, 334*, 146-162.

DelProposto, J., Majmudar, C. Y., Smith, J. L., & Brown, W. C. (2009). Mocr: A novel fusion tag for enhancing solubility that is compatible with structural biology applications. *Protein Expression and Purification, 63*(1), 40-49. doi:10.1016/j.pep.2008.08.011; 10.1016/j.pep.2008.08.011

Depew, R. E., Liu, L. F., & Wang, J. C. (1978). Interaction between DNA and *Escherichia coli* protein omega. Formation of a complex between single-stranded DNA and omega protein. *The Journal of Biological Chemistry, 253*(2), 511-518.

Doug Sweetser, Michael Nonet, & Richard A. Young. (1987). Prokaryotic and eukaryotic RNA polymerases have homologous core subunits. *Proceedings of the National Academy of Sciences of the United States of America, 84*(5), 1192-1196.

Dörr, T., Lewis, K., & Vulić, M. (2009). SOS response induces persistence to fluoroquinolones in *Escherichia coli*. *PLoS Genetics, 5*(12), e1000760. doi:10.1371/journal.pgen.1000760

Doyle, S. A. (2005). High-throughput cloning for proteomics research. *Methods in Molecular Biology (Clifton, N.J.), 310*, 107-113.

Drlica, K. (1992). Control of bacterial DNA supercoiling. *Molecular Microbiology, 6*(4), 425-433.

Drolet, M., Phoenix, P., Menzel, R., Massé, E., Liu, L. F., & Crouch, R. J. (1995). Overexpression of RNase H partially complements the growth defect of an *Escherichia coli* delta topA mutant: R-loop formation is a major problem in the absence of DNA topoisomerase I. *Proceedings of the National Academy of Sciences, 92*(8), 3526-3530. doi:10.1073/pnas.92.8.3526

Duguet, M. (1997). When helicase and topoisomerase meet! *Journal of Cell Science, 110*, 1345-1350.

Duhovny, D., Nussinov, R., & Wolfson, H. J. (2002/9/17). Efficient unbound docking of rigid molecules. Paper presented at the International Workshop on the Algorithms in Bioinformatics. 185-200. doi:10.1007/3-540-45784-4_14

Duncan E. Scott, Matthias T. Ehebauer, Tara Pukala, May Marsh, Tom L. Blundell, Ashok R. Venkitaraman, . . . Marko Hyvönen. (2013). Using a fragment-based approach to target protein-protein interactions. *ChemBioChem, 14*(3), 332-342.

Duncan E. Scott, Matthias T. Ehebauer, Tara Pukala, May Marsh, Tom L. Blundell, Ashok R. Venkitaraman, . . . Marko Hyvönen. (2013). Using a fragment-based approach to target protein-protein interactions. *ChemBioChem, 14*(3), 332-342. doi:10.1002/cbic.201200521

Ebright, R. H. (2000). RNA polymerase: Structural similarities between bacterial RNA polymerase and eukaryotic RNA polymerase II. *Journal of Molecular Biology, 304*(5), 687-698. doi:10.1006/jmbi.2000.4309

Edmondson, D. G., & Dent, S. Y. R. (2001). Identification of protein interactions by far western analysis. *Current protocols in protein science* () John Wiley & Sons, Inc. Retrieved from http://onlinelibrary.wiley.com/doi/10.1002/0471140864.ps1907s25/abstract

Elass, E., Coddeville, B., Guérardel, Y., Kremer, L., Maes, E., Mazurier, J., & Legrand, D. (2007). Identification by surface plasmon resonance of the mycobacterial lipomannan and lipoarabinomannan domains involved in binding to CD14 and LPS-binding protein. *FEBS Letters, 581*(7), 1383-1390. doi:10.1016/j.febslet.2007.02.056

Ellis, N. A., Groden, J., Ye, T. Z., Straughen, J., Lennon, D. J., Ciocci, S., . . . German, J. (1995). The bloom's syndrome gene product is homologous to RecQ helicases. *Cell, 83*(4), 655-666.

Esposito, D., & Chatterjee, D. K. (2006). Enhancement of soluble protein expression through the use of fusion tags. *Current Opinion in Biotechnology, 17*(4), 353-358.

Feklistov, A. (2013). RNA polymerase: In search of promoters. *Annals of the New York Academy of Sciences, 1293*(1), 25-32. doi:10.1111/nyas.12197

Ferrándiz, M., & de la Campa, Adela G. (2014). The fluoroquinolone levofloxacin triggers the transcriptional activation of iron transport genes that contribute to cell death in *Streptococcus pneumoniae*. *Antimicrobial Agents and Chemotherapy, 58*(1), 247-257. doi:10.1128/AAC.01706-13

Fiorani, P., Amatruda, J. F., Silvestri, A., Butler, R. H., Bjornsti, M. A., & Benedetti, P. (1999). Domain interactions affecting human DNA topoisomerase I catalysis and camptothecin sensitivity. *Molecular Pharmacology, 56*(6), 1105-1115.

Forterre, P., Gribaldo, S., Gadelle, D., & Serre, M. (2007). Origin and evolution of DNA topoisomerases. *Biochimie, 89*(4), 427-446. doi:10.1016/j.biochi.2006.12.009

Fortune, J. M., & Osheroff, N. (2000). Topoisomerase II as a target for anticancer drugs: When enzymes stop being nice. *Progress in Nucleic Acid Research and Molecular Biology, 64*, 221-253.

Free, R. B., Hazelwood, L. A., & Sibley, D. R. (2009). Identifying novel protein-protein interactions using co-immunoprecipitation and mass spectroscopy. *Current Protocols in Neuroscience, Chapter 5*, Unit 5.28. doi:10.1002/0471142301.ns0528s46

Froger, A., & Hall, J. E. (2007). Transformation of plasmid DNA into *E. coli* using the heat shock method. *Journal of Visualized Experiments : JoVE, (6):253. doi*(6), 253. doi:10.3791/253; 10.3791/253

Frøhlich, R. F., Veigaard, C., Andersen, F. F., McClendon, A. K., Gentry, A. C., Andersen, A. H., . . . Knudsen, B. R. (2007). Tryptophane-205 of human topoisomerase I is essential for camptothecin inhibition of negative but not positive supercoil removal. *Nucleic Acids Research, 35*(18), 6170-6180. doi:10.1093/nar/gkm669

Gangloff, S., McDonald, J. P., Bendixen, C., Arthur, L., & Rothstein, R. (1994). The yeast type I topoisomerase Top3 interacts with Sgs1, a DNA helicase homolog: A potential eukaryotic reverse gyrase. *Molecular and Cellular Biology, 14*(12), 8391-8398.

Gellert, M., Mizuuchi, K., O'Dea, M. H., & Nash, H. A. (1976). DNA gyrase: An enzyme that introduces superhelical turns into DNA. *Proceedings of the National Academy of Sciences, 73*(11), 3872-3876.

German, J. (1997). Bloom's syndrome. XX. the first 100 cancers. *Cancer Genetics and Cytogenetics, 93*(1), 100-106.

Gibson, D. G. (2011). Enzymatic assembly of overlapping DNA fragments. *Methods in Enzymology, 498*, 349-361. doi:10.1016/B978-0-12-385120-8.00015-2

Ghosh, S., Mallick, B., & Nagaraja, V. (2014). Direct regulation of topoisomerase activity by a nucleoid-associated protein. *Nucleic Acids Research, 42*(17), 11156-11165. doi:10.1093/nar/gku804 [doi]

Ginsburg, A. S., Grosset, J. H., & Bishai, W. R. (2003). Fluoroquinolones, tuberculosis, and resistance. *The Lancet Infectious Diseases, 3*(7), 432-442. doi:10.1016/S1473-3099(03)00671-6

Godbole, A. A., Ahmed, W., Bhat, R. S., Bradley, E. K., Ekins, S., & Nagaraja, V. (2015). Targeting mycobacterium tuberculosis topoisomerase I by small-molecule inhibitors. *Antimicrobial Agents and Chemotherapy, 59*(3), 1549-1557. doi:10.1128/AAC.04516-14

Goldberg, D. E., Siliciano, R. F., & Jacobs, W. R.,Jr. (2012). Outwitting evolution: Fighting drug-resistant TB, malaria, and HIV. *Cell, 148*(6), 1271-1283. doi:10.1016/j.cell.2012.02.021 [doi]

Golemis, E. (2002). *Protein-protein interactions: A molecular cloning manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Goulding, C. W., & Perry, L. J. (2003). Protein production in *Escherichia coli* for structural studies by X-ray crystallography. *Journal of Structural Biology, 142*(1), 133-143.

Gray, J. J., Moughon, S., Wang, C., Schueler-Furman, O., Kuhlman, B., Rohl, C. A., & Baker, D. (2003). Protein-protein docking with simultaneous optimization of rigid-body displacement and side-chain conformations. *Journal of Molecular Biology, 331*(1), 281-299.

Grishin, N. V. (2000). C-terminal domains of *Escherichia coli* topoisomerase I belong to the zinc-ribbon superfamily. *Journal of Molecular Biology, 299*(5), 1165-1177.

Günther, G. (2014). Multidrug-resistant and extensively drug-resistant tuberculosis: A review of current concepts and future challenges. *Clinical Medicine (London, England), 14*(3), 279-285. doi:10.7861/clinmedicine.14-3-279

Hanai, R., Caron, P. R., & Wang, J. C. (1996). Human TOP3: A single-copy gene encoding DNA topoisomerase III. *Proceedings of the National Academy of Sciences of the United States of America, 93*(8), 3653-3657.

Harmon, F. G., Rehrauer, W. M., & Kowalczykowski, S. C. (1996). Interaction of *Escherichia coli* RecA protein with LexA repressor. II. inhibition of DNA strand exchange by the uncleavable LexA S119A repressor argues that recombination and SOS induction are competitive processes. *The Journal of Biological Chemistry, 271*(39), 23874-23883.

Hartsuiker, E., Bahler, J., & Kohli, J. (1998). The role of topoisomerase II in meiotic chromosome condensation and segregation in *Schizosaccharomyces pombe*. *Molecular Biology of the Cell, 9*(10), 2739-2750.

Hastings, P. J., Rosenberg, S. M., & Slack, A. (2004). Antibiotic-induced lateral transfer of antibiotic resistance. *Trends in Microbiology, 12*(9), 401-404.

Hedren, M., Ballagi, A., Mortsell, L., Rajkai, G., Stenmark, P., Sturesson, C., & Nordlund, P. (2006). GRETA, a new multifermenter system for structural genomics and process optimization. *Acta Crystallographica.Section D, Biological Crystallography, 62*(Pt 10), 1227-1231. doi:10.1107/S090744490603441X

Hoatlin, M., Yin, J., Sobeck, A., Li, L., Meetei, A. R., Xu, C., & Wang, W. (2005). BLAP75, an essential component of bloom's syndrome protein complexes that maintain genome integrity. *The EMBO Journal, 24*(7), 1465-1476.

Howard-Flanders, P., West, S. C., & Stasiak, A. (1984). Role of RecA protein spiral filaments in genetic recombination. *Nature, 309*(5965), 215-219.

Hraiky, C., Raymond, M. A., & Drolet, M. (2000). RNase H overproduction corrects a defect at the level of transcription elongation during rRNA synthesis in the absence of DNA topoisomerase I in *Escherichia coli*. *The Journal of Biological Chemistry, 275*(15), 11257-11263.

Hu, P., Beresten, S. F., van Brabant, A. J., Ye, T. Z., Pandolfi, P. P., Johnson, F. B., . . . Ellis, N. A. (2001). Evidence for BLM and topoisomerase III alpha interaction in genomic stability. *Human Molecular Genetics, 10*(12), 1287-1298.

Huang, F., & He, Z. (2010). Characterization of an interplay between a *Mycobacterium tuberculosis* MazF homolog, Rv1495 and its sole DNA topoisomerase I. *Nucleic Acids Research, 38*(22), 8219-8230. doi:10.1093/nar/gkq737

Humphrey, W., Dalke, A., & Schulten, K. (1996). VMD: Visual molecular dynamics. *Journal of Molecular Graphics, 14*(1), 28.

Iwabata, K., Koshiyama, A., Yamaguchi, T., Sugawara, H., Hamada, F. N., Namekawa, S. H., . . . Sakaguchi, K. (2005). DNA topoisomerase II interacts with Lim15/Dmc1 in meiosis. *Nucleic Acids Research, 33*(18), 5809-5818. doi:10.1093/nar/gki883

Janet R. Mullen, Ferez S. Nallaseth, Yan Q. Lan, Christopher E. Slagle, & Steven J. Brill. (2005). Yeast Rmi1/Nce4 controls genome stability as a subunit of the Sgs1-Top3 complex. *Molecular and Cellular Biology, 25*(11), 4476-4487. doi:10.1128/MCB.25.11.4476-4487.2005

Jayanthi, S., Morris, J., Kachel, B., Al-Ameer, M., Henderson, R., Adams, P. D., & Kumar, T. K. S. (2015). The versatility of isothermal titration calorimetry in modern biology. *Journal of Analytical & Bioanalytical Techniques, 6*(3) doi:10.4172/2155-9872.1000e121

Jiménez-García, B., Pons, C., & Fernández-Recio, J. (2013). pyDockWEB: A web server for rigid-body protein-protein docking using electrostatics and desolvation scoring. *Bioinformatics (Oxford, England), 29*(13), 1698-1699. doi:10.1093/bioinformatics/btt262

Johnson, F. B., Lombard, D. B., Neff, N. F., Mastrangelo, M., Dewolf, W., Ellis, N. A., . . . Guarente, L. (2000). Association of the bloom syndrome protein with topoisomerase IIIα in somatic and meiotic cells. *Cancer Research, 60*(5), 1162-1167.

Jönsson, U., Fägerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., . . . Rönnberg, I. (1991). Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *BioTechniques, 11*(5), 620-627.

K, T., N, C., B, C., A, J., & Yc, T. (2016). Insights from the structure of *Mycobacterium tuberculosis* topoisomerase I with a novel protein fold. *Journal of Molecular Biology, Journal of Molecular Biology, 428, 428*(1, 1), 193.

Karayan, L., Riou, J. F., Séité, P., Migeon, J., Cantereau, A., & Larsen, C. J. (2001). Human ARF protein interacts with topoisomerase I and stimulates its activity. *Oncogene, 20*(7), 836-848. doi:10.1038/sj.onc.1204170

Karlin, S., & Brocchieri, L. (1996). Evolutionary conservation of RecA genes in relation to protein structure and function. *Journal of Bacteriology, 178*(7), 1881-1894.

Karplus, M., & McCammon, J. A. (2002). Molecular dynamics simulations of biomolecules. *Nature Structural Biology, 9*(9), 646-652. doi:10.1038/nsb0902-646

Kastritis, P. L., & Bonvin, Alexandre M J J. (2013). On the binding affinity of macromolecular interactions: Daring to ask why proteins interact. *Journal of the Royal Society, Interface, 10*(79), 20120835. doi:10.1098/rsif.2012.0835

Katie J Aldred, Robert J Kerns, & Neil Osheroff. (2014). *Mechanism of quinolone action and resistance*. United States: American Chemical Society. doi:10.1021/bi5000564

Kaufman, C. S., Genovese, A., & Butler, M. G. (2016). Deletion of TOP3B is associated with cognitive impairment and facial dysmorphism. *Cytogenetic and Genome Research, 150*(2), 106-111. doi:10.1159/000452815

Kawasaki, K., Minoshima, S., Nakato, E., Shibuya, K., Shintani, A., Schmeits, J. L., . . . Shimizu, N. (1997). One-megabase sequence analysis of the human immunoglobulin lambda gene locus. *Genome Research, 7*(3), 250-261.

Kikuchi, A., & Asai, K. (1984). Reverse gyrase—a topoisomerase which introduces positive superhelical turns into DNA. *Nature, 309*(5970), 677-681. doi:10.1038/309677a0

Kowalska-Loth, B., Girstun, A., Trzcińska, A. M., Piekiełko-Witkowska, A., & Staroń, K. (2005). SF2/ASF protein binds to the cap region of human topoisomerase I through two RRM domains. *Biochemical and Biophysical Research Communications, 331*(2), 398-403. doi:10.1016/j.bbrc.2005.03.180

Krogh, B. O., & Shuman, S. (2002). A poxvirus-like type IB topoisomerase family in bacteria. *Proceedings of the National Academy of Sciences of the United States of America, 99*(4), 1853-1858. doi:10.1073/pnas.032613199

Konola, J. T., Logan, K. M., & Knight, K. L. (1994). Functional characterization of residues in the P-loop motif of the RecA protein ATP binding site. *Journal of Molecular Biology, 237*(1), 20-34. doi:10.1006/jmbi.1994.1206

Kuo, W. H., & Chase, H. A. (2011). Exploiting the interactions between poly-histidine fusion tags and immobilized metal ions. *Biotechnology Letters, 33*(6), 1075-1084. doi:10.1007/s10529-011-0554-3; 10.1007/s10529-011-0554-3

Kwan, K. Y., & Wang, J. C. (2001). Mice lacking DNA topoisomerase IIIbeta develop to maturity but show a reduced mean lifespan. *Proceedings of the National Academy of Sciences of the United States of America, 98*(10), 5717-5721.

Kwan, K. Y., Moens, P. B., & Wang, J. C. (2003). Infertility and aneuploidy in mice lacking a type IA DNA topoisomerase III beta. *Proceedings of the National Academy of Sciences of the United States of America, 100*(5), 2526-2531.

Laskowski, R. A. (2001). PDBsum: Summaries and analyses of PDB structures. *Nucleic Acids Research, 29*(1), 221-222.

Laskowski, R. A., Hutchinson, E. G., Michie, A. D., Wallace, A. C., Jones, M. L., & Thornton, J. M. (1997). PDBsum: A web-based database of summaries and analyses of all PDB structures. *Trends in Biochemical Sciences, 22*(12), 488-490.

Lee, C., Hague, L. K., Li, H., & Donnelly, R. (2004). Identification of toposome, a novel multisubunit complex containing topoisomerase II alpha. *Cell Cycle (Georgetown, Tex.), 3*(5), 638-647.

Lee, J., Cheng, X., Swails, J. M., Yeom, M. S., Eastman, P. K., Lemkul, J. A., . . . Im, W. (2016). CHARMM-GUI input generator for NAMD, GROMACS, AMBER, OpenMM, and CHARMM/OpenMM simulations using the CHARMM36 additive force field. *Journal of Chemical Theory and Computation, 12*(1), 405-413.

Li, W., & Wang, J. C. (1998). Mammalian DNA topoisomerase III alpha is essential in early embryogenesis. *Proceedings of the National Academy of Sciences of the United States of America, 95*(3), 1010-1013.

Lima, C. D., Wang, J. C., & Mondragón, A. (1994). Three-dimensional structure of the 67K N-terminal fragment of *E. coli* DNA topoisomerase I. *Nature, 367*(6459), 138-146. doi:10.1038/367138a0

Liang, W., Malhotra, A., & Deutscher, M. P. (2011). Acetylation regulates the stability of a bacterial protein: Growth stage-dependent modification of RNase R. *Molecular Cell, 44*(1), 160-166. doi:10.1016/j.molcel.2011.06.037 [doi]

Lin, Z., Kong, H., Nei, M., & Ma, H. (2006). Origins and evolution of the recA/RAD51 gene family: Evidence for ancient gene duplication and endosymbiotic gene transfer. *Proceedings of the National Academy of Sciences of the United States of America, 103*(27), 10328-10333. doi:0604232103 [pii]

Little, J. W. (1991). Mechanism of specific LexA cleavage: Autodigestion and the role of RecA coprotease. *Biochimie, 73*(4), 411-421.

Liu, L. F., & Wang, J. C. (1987). Supercoiling of the DNA template during transcription. *Proceedings of the National Academy of Sciences of the United States of America, 84*(20), 7024-7027.

Liu, I. -., Sutherland, J. H., Cheng, B., & Tse-Dinh, Y. (2011). Topoisomerase I function during *Escherichia coli* response to antibiotics and stress enhances cell killing from stabilization of its cleavage complex. *The Journal of Antimicrobial Chemotherapy, 66*(7), 1518-1524. doi:10.1093/jac/dkr150

Los, G. V., Encell, L. P., McDougall, M. G., Hartzell, D. D., Karassina, N., Zimprich, C., . . . Wood, K. V. (2008). HaloTag: A novel protein labeling technology for cell imaging and protein analysis. *ACS Chemical Biology, 3*(6), 373-382.

Lusetti, S. L., & Cox, M. M. (2002). The bacterial RecA protein and the recombinational DNA repair of stalled replication forks. *Annual Review of Biochemistry, 71*, 71-100. doi:10.1146/annurev.biochem.71.083101.133940 [doi]

Lynn, R. M., & Wang, J. C. (1989). Peptide sequencing and site-directed mutagenesis identify tyrosine-319 as the active site tyrosine of *Escherichia coli* DNA topoisomerase I. *Proteins, 6*(3), 231-239. doi:10.1002/prot.340060305

Lu, J., Mullen, J. R., Brill, S. J., Kleff, S., Romeo, A. M., & Sternglanz, R. (1996). Human homologues of yeast helicase. *Nature, 383*(6602), 678-679.

Ma, J et al., (2013). Transcription under torsion. *Science*, 340(6140), 5180-5183.

MacDonald, T. L., & Legnert, E. K. (1991). *DNA topoisomerases in cancer* Oxford University Press.

Madden, K. R., Stewart, L., & Champoux, J. J. (1995). Preferential binding of human topoisomerase I to superhelical DNA. *The EMBO Journal, 14*(21), 5399-5409.

Madiraju, M., Madiraju, S. C., Yamamoto, K., Greendyke, R., & Rajagopalan, M. (2011). Replacement of *Mycobacterium smegmatis* dnaA gene by *Mycobacterium tuberculosis* homolog results in temperature sensitivity. *Tuberculosis (Edinburgh, Scotland), 91 Suppl 1*, 136. doi:10.1016/j.tube.2011.10.023 [doi]

Mahmood, T., & Yang, P. C. (2012). Western blot: Technique, theory, and trouble shooting. *North American Journal of Medical Sciences, 4*(9), 429-434.

Malik, M., Lu, T., Zhao, X., Singh, A., Hattan, C. M., Domagala, J., . . . Drlica, K. (2005). Lethality of quinolones against *Mycobacterium smegmatis* in the presence or absence of chloramphenicol. *Antimicrobial Agents and Chemotherapy, 49*(5), 2008-2014. doi:49/5/2008 [pii]

Malik, M., Marks, K. R., Mustaev, A., Zhao, X., Chavda, K., Kerns, R. J., & Drlica, K. (2011). Fluoroquinolone and quinazolinedione activities against wild-type and gyrase mutant strains of *Mycobacterium smegmatis*. *Antimicrobial Agents and Chemotherapy, 55*(5), 2335-2343. doi:10.1128/AAC.00033-11 [doi]

Mankouri, H. W., & Hickson, I. D. (2007). The RecQ helicase–topoisomerase III–Rmi1 complex: A DNA structure-specific 'dissolvasome'? *Trends in Biochemical Sciences, 32*(12), 538-546. doi:10.1016/j.tibs.2007.09.009

Marceau, A. H., Bernstein, D. A., Walsh, B. W., Shapiro, W., Simmons, L. A., & Keck, J. L. (2013). Protein interactions in genome maintenance as novel antibacterial targets. *PloS One, 8*(3), e58765. doi:10.1371/journal.pone.0058765 [doi]

Mashiach, E., Schneidman-Duhovny, D., Andrusier, N., Nussinov, R., & Wolfson, H. J. (2008). FireDock: A web server for fast interaction refinement in molecular docking. *Nucleic Acids Research, 36*(Web Server issue), 229.

Massé, E., & Drolet, M. (1999a). Relaxation of transcription-induced negative supercoiling is an essential function of *Escherichia coli* DNA topoisomerase I. *The Journal of Biological Chemistry, 274*(23), 16654-16658.

Massé, E., & Drolet, M. (1999b). *Escherichia coli* DNA topoisomerase I inhibits R-loop formation by relaxing transcription-induced negative supercoiling. *The Journal of Biological Chemistry, 274*(23), 16659-16664.

Maxwell, A., & Gellert, M. (1986). Mechanistic aspects of DN topoisomerases. *Advances in Protein Chemistry, 38*, 69-107. doi:10.1016/S0065-3233(08)60526-4

McClendon, A. K., & Osheroff, N. (2007). DNA topoisomerase II, genotoxicity, and cancer. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 623*(1), 83-97. doi:10.1016/j.mrfmmm.2007.06.009

McGrew, D. A., & Knight, K. L. (2003). Molecular design and functional organization of the RecA protein. *Critical Reviews in Biochemistry and Molecular Biology, 38*(5), 385-432. doi:10.1080/10409230390242489 [doi]

Minami, Y., Kawasaki, H., Minami, M., Tanahashi, N., Tanaka, K., & Yahara, I. (2000). A critical role for the proteasome activator PA28 in the Hsp90-dependent protein refolding. *The Journal of Biological Chemistry, 275*(12), 9055-9061.

Nagaraja, V., Sikder, D., & Jain, P. (2002). DNA topoisomerase I from mycobacteria--a potential drug target. *Current Pharmaceutical Design, 8*(22), 1995-2007.

Nagaraja, V., Godbole, A. A., Henderson, S. R., & Maxwell, A. (2017). DNA topoisomerase I and DNA gyrase as targets for TB therapy. *Drug Discovery Today,* doi:10.1016/j.drudis.2016.11.006

Nallamsetty, S., & Waugh, D. S. (2006). Solubility-enhancing proteins MBP and NusA play a passive role in the folding of their fusion partners. *Protein Expression and Purification, 45*(1), 175-182. doi:S1046-5928(05)00224-X [pii]

Narula, G., Annamalai, T., Aedo, S., Cheng, B., Sorokin, E., Wong, A., & Tse-Dinh, Y. (2011). The strictly conserved arg-321 residue in the active site of *Escherichia coli* topoisomerase I plays a critical role in DNA rejoining. *The Journal of Biological Chemistry, 286*(21), 18673-18680. doi:10.1074/jbc.M111.229450

Ngounou Wetie, A. G., Sokolowska, I., Woods, A. G., Roy, U., Loo, J. A., & Darie, C. C. (2013). Investigation of stable and transient protein-protein interactions: Past, present, and future. *Proteomics, 13*(3-4), 538-557.

Nimesh, H., Sur, S., Sinha, D., Yadav, P., Anand, P., Bajaj, P., . . . Tandon, V. (2014). Synthesis and biological evaluation of novel bisbenzimidazoles as *Escherichia coli* topoisomerase IA inhibitors and potential antibacterial agents. *Journal of Medicinal Chemistry, 57*(12), 5238-5257. doi:10.1021/jm5003028

Nitiss, J. L. (1998). Investigating the biological functions of DNA topoisomerases in eukaryotic cells. *Biochimica Et Biophysica Acta, 1400*(1-3), 63-81.

Opalka, N., Brown, J., Lane, W. J., Twist, K. F., Landick, R., Asturias, F. J., & Darst, S. A. (2010). Complete structural model of *Escherichia coli* RNA polymerase from a hybrid approach. *PLoS Biology, 8*(9) doi:10.1371/journal.pbio.1000483

Osheroff, N., Corbett, A. H., Elsea, S. H., & Westergaard, M. (1994). Defining functional drug-interaction domains on topoisomerase II by exploiting mechanistic differences between drug classes. *Cancer Chemotherapy and Pharmacology, 34 Suppl*, 19.

Otsuki, M., Seki, M., Inoue, E., Abe, T., Narita, Y., Yoshimura, A., . . . Enomoto, T. (2008). Analyses of functional interaction between RECQL1, RECQL5, and BLM which physically interact with DNA topoisomerase IIIalpha. *Biochimica Et Biophysica Acta, 1782*(2), 75-81. doi:10.1016/j.bbadis.2007.11.003

Papadopoulos, J. S., & Agarwala, R. (2007). COBALT: Constraint-based alignment tool for multiple protein sequences. *Bioinformatics (Oxford, England), 23*(9), 1073-1079. doi:10.1093/bioinformatics/btm076

Parks, T. D., Leuther, K. K., Howard, E. D., Johnston, S. A., & Dougherty, W. G. (1994). Release of proteins and peptides from fusion proteins using a recombinant plant virus proteinase. *Analytical Biochemistry, 216*(2), 413-417.

Pashley, C. A., Parish, T., McAdam, R. A., Duncan, K., & Stoker, N. G. (2003). Gene replacement in mycobacteria by using incompatible plasmids. *Applied and Environmental Microbiology, 69*(1), 517-523.

Patel, M., Jiang, Q., Woodgate, R., Cox, M. M., & Goodman, M. F. (2010). A new model for SOS-induced mutagenesis: How RecA protein activates DNA polymerase V. *Critical Reviews in Biochemistry and Molecular Biology, 45*(3), 171-184. doi:10.3109/10409238.2010.480968 [doi]

Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., & Ferrin, T. E. (2004). UCSF chimera--a visualization system for exploratory research and analysis. *Journal of Computational Chemistry, 25*(13), 1605-1612.

Pelicic, V., Jackson, M., Reyrat, J. M., Jacobs, W. R.,Jr, Gicquel, B., & Guilhot, C. (1997). Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America, 94*(20), 10955-10960.

Perkins, J. R., Diboun, I., Dessailly, B. H., Lees, J. G., & Orengo, C. (2010). Transient protein-protein interactions: Structural, functional, and network properties. *Structure (London, England : 1993), 18*(10), 1233-1243.

Phan, J., Zdanov, A., Evdokimov, A. G., Tropea, J. E., Peters, H. K., Kapust, R. B., . . . Waugh, D. S. (2002). Structural basis for the substrate specificity of tobacco etch virus protease. *Journal of Biological Chemistry, 277*(52), 50564-50572.

Phillips, J. C., Braun, R., Wang, W., Gumbart, J., Tajkhorshid, E., Villa, E., . . . Schulten, K. (2005). Scalable molecular dynamics with NAMD. *Journal of Computational Chemistry, 26*(16), 1781-1802. doi:10.1002/jcc.20289

Phizicky, E. M., & Fields, S. (1995). Protein-protein interactions: Methods for detection and analysis. *Microbiological Reviews, 59*(1), 94-123.

Pierce, M. M., Raman, C. S., & Nall, B. T. (1999). Isothermal titration calorimetry of Protein–Protein interactions. *Methods, 19*(2), 213-221. doi:10.1006/meth.1999.0852

Plocinski, P., Laubitz, D., Cysewski, D., Stodus, K., Kowalska, K., & Dziembowski, A. (2014). Identification of protein partners in mycobacteria using a single-step affinity purification method. *PloS One, 9*(3), e91380. doi:10.1371/journal.pone.0091380

Płociński, P., Laubitz, D., Cysewski, D., Stoduś, K., Kowalska, K., & Dziembowski, A. (2014). Identification of protein partners in mycobacteria using a single-step affinity purification method. *PloS One, 9*(3), e91380. doi:10.1371/journal.pone.0091380

Pommier, Y. (1997). DNA topoisomerase II inhibitors. In B. A. Teicher (Ed.), *Cancer therapeutics* (pp. 153-174) Humana Press.

Pommier, Y. (2013). Drugging topoisomerases: Lessons and challenges. *ACS Chemical Biology, 8*(1), 82-95. doi:10.1021/cb300648v

Pommier, Y., Leo, E., Zhang, H., & Marchand, C. (2010). DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chemistry & Biology, 17*(5), 421-433. doi:10.1016/j.chembiol.2010.04.012

Pommier, Y., & Marchand, C. (2005). Interfacial inhibitors of protein-nucleic acid interactions. *Current Medicinal Chemistry. Anti-Cancer Agents, 5*(4), 421-429.

Ranjan, N., Fulcrand, G., King, A., Brown, J., Jiang, X., Leng, F., & Arya, D. P. (2014). Selective inhibition of bacterial topoisomerase I by alkynyl-bisbenzimidazoles. *MedChemComm, 5*(6), 816. doi:10.1039/c4md00140k

Ravishankar, S., Ambady, A., Awasthy, D., Mudugal, N. V., Menasinakai, S., Jatheendranath, S., . . . Sharma, U. K. (2015). Genetic and chemical validation identifies *Mycobacterium tuberculosis* topoisomerase I as an attractive anti-tubercular target. *Tuberculosis, 95*(5), 589-598. doi:10.1016/j.tube.2015.05.004

Ray-Soni, A., Bellecourt, M. J., & Landick, R. (2016). Mechanisms of bacterial transcription termination: All good things must end. *Annual Review of Biochemistry, 85*(1), 319-347. doi:10.1146/annurev-biochem-060815-014844

Reckinger, A. R., Jeong, K. S., Khodursky, A. B., & Hiasa, H. (2007). RecA can stimulate the relaxation activity of topoisomerase I: Molecular basis of topoisomerase-mediated genome-wide transcriptional responses in *Escherichia coli*. *Nucleic Acids Research, 35*(1), 79-86. doi:gkl981 [pii]

Redinbo, M. R., Stewart, L., Kuhn, P., Champoux, J. J., & Hol, W. G. (1998). Crystal structures of human topoisomerase I in covalent and noncovalent complexes with DNA. *Science (New York, N.Y.), 279*(5356), 1504-1513.

Sandhaus, S., Annamalai, T., Welmaker, G., Houghten, R. A., Paz, C., Garcia, P. K., . . . Tse-Dinh, Y. (2016). Small-molecule inhibitors targeting topoisomerase I as novel antituberculosis agents. *Antimicrobial Agents and Chemotherapy, 60*(7), 4028-4036.

Sassetti, C. M., & Rubin, E. J. (2003). Genetic requirements for mycobacterial survival during infection. *Proceedings of the National Academy of Sciences of the United States of America, 100*(22), 12989-12994. doi:10.1073/pnas.2134250100

Schneidman-Duhovny, D., Inbar, Y., Nussinov, R., & Wolfson, H. J. (2005). PatchDock and SymmDock: Servers for rigid and symmetric docking. *Nucleic Acids Research, 33*(Web Server issue), 363. doi:10.1093/nar/gki481

Schoenrock, A., Burnside, D., Moteshareie, H., Pitre, S., Hooshyar, M., Green, J. R., . . . Wong, A. (2017). Evolution of protein-protein interaction networks in yeast. *PloS One, 12*(3), e0171920. doi:10.1371/journal.pone.0171920

Scott, D. E., Coyne, A. G., Hudson, S. A., & Abell, C. (2012). Fragment-based approaches in drug discovery and chemical biology. *Biochemistry, 51*(25), 4990-5003. doi:10.1021/bi3005126 [doi]

Serre, M., & Duguet, M. (2003). Enzymes that cleave and religate DNA at high temperature: The same story with different actors. *Progress in Nucleic Acid Research and Molecular Biology, 74*, 37-81.

Shiloh, M. U., & Champion, P. A. D. (2010). To catch a killer. what can mycobacterial models teach us about *Mycobacterium tuberculosis* pathogenesis? *Current Opinion in Microbiology, 13*(1), 86-92. doi:10.1016/j.mib.2009.11.006

Shvetsov, A. V., Lebedev, D. V., Chervyakova, D. B., Bakhlanova, I. V., Yung, I. A., Radulescu, A., . . . Isaev-Ivanov, V. V. (2014). Structure of RecX protein complex with the presynaptic RecA filament: Molecular dynamics simulations and small angle neutron scattering. *FEBS Letters, 588*(6), 948-955.

Singh, W., Karabencheva-Christova, T. G., Black, G. W., Ainsley, J., Dover, L., & Christov, C. Z. (2016). Conformational dynamics, ligand binding and effects of mutations in NirE an S-adenosyl-L-methionine dependent methyltransferase. *Scientific Reports, 6*, 20107. doi:10.1038/srep20107

Slesarev, A. I., Stetter, K. O., Lake, J. A., Gellert, M., Krah, R., & Kozyavkin, S. A. (1993). DNA topoisomerase V is a relative of eukaryotic topoisomerase I from a hyperthermophilic prokaryote. *Nature, 364*(6439), 735-737. doi:10.1038/364735a0

Smith, G. R., & Sternberg, M. J. E. (2002). Prediction of protein-protein interactions by docking methods. *Current Opinion in Structural Biology, 12*(1), 28-35.

Sorokin, E. P., Cheng, B., Rathi, S., Aedo, S. J., Abrenica, M. V., & Tse-Dinh, Y. C. (2008). Inhibition of Mg^{2+} binding and DNA religation by bacterial topoisomerase I via introduction of an additional positive charge into the active site region. *Nucleic Acids Research, 36*(14), 4788-4796. doi:10.1093/nar/gkn460 [doi]

Sousa, M. M., Steen, K. W., Hagen, L., & Slupphaug, G. (2011). Antibody cross-linking and target elution protocols used for immunoprecipitation significantly modulate signal-to noise ratio in downstream 2D-PAGE analysis. *Proteome Science, 9*, 45.

Stockum, A., Lloyd, R. G., & Rudolph, C. J. (2012). On the viability of *Escherichia coli* cells lacking DNA topoisomerase I. *BMC Microbiology, 12*, 26. doi:10.1186/1471-2180-12-26

Stoll, G., Pietiläinen, O. P. H., Linder, B., Suvisaari, J., Brosi, C., Hennah, W., . . . Palotie, A. (2013). Deletion of TOP3β, a component of FMRP-containing mRNPs, contributes to neurodevelopmental disorders. *Nature Neuroscience, 16*(9), 1228-1237. doi:10.1038/nn.3484

Story, R. M., & Steitz, T. A. (1992). Structure of the recA protein-ADP complex. *Nature, 355*(6358), 374-376. doi:10.1038/355374a0

Stupina, V. A., & Wang, J. C. (2005). Viability of *Escherichia coli* topA mutants lacking DNA topoisomerase I. *The Journal of Biological Chemistry, 280*(1), 355-360.

Suerbaum, S., Brauer-Steppkes, T., Labigne, A., Cameron, B., & Drlica, K. (1998). Topoisomerase I of helicobacter pylori: Juxtaposition with a flagellin gene (flaB) and functional requirement of a fourth zinc finger motif. *Gene, 210*(1), 151-161.

Sun, H., Karow, J. K., Hickson, I. D., & Maizels, N. (1998). The bloom's syndrome helicase unwinds G4 DNA. *The Journal of Biological Chemistry, 273*(42), 27587-27592.

Szafran, M., Skut, P., Ditkowski, B., Ginda, K., Chandra, G., Zakrzewska-Czerwińska, J., & Jakimowicz, D. (2013). Topoisomerase I (TopA) is recruited to ParB complexes and is required for proper chromosome organization during *Streptomyces coelicolor* sporulation. *Journal of Bacteriology, 195*(19), 4445-4455. doi:10.1128/JB.00798-13

Tan, K., Zhou, Q., Cheng, B., Zhang, Z., Joachimiak, A., & Tse-Dinh, Y. (2015). Structural basis for suppression of hypernegative DNA supercoiling by *E. coli* topoisomerase I. *Nucleic Acids Research, 43*(22), 11031-11046. doi:10.1093/nar/gkv1073

Terekhova, K., Marko, J. F., & Mondragón, A. (2013). Studies of bacterial topoisomerases I and III at the single molecule level. *Biochemical Society Transactions, 41*(2), 571-575. doi:10.1042/BST20120297

Terpe, K. (2003). Overview of tag protein fusions: From molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology, 60*(5), 523-533. doi:10.1007/s00253-002-1158-6

Thi, T. D., López, E., Rodríguez-Rojas, A., Rodríguez-Beltrán, J., Couce, A., Guelfo, J. R., . . . Blázquez, J. (2011). Effect of recA inactivation on mutagenesis of *Escherichia coli* exposed to sublethal concentrations of antimicrobials. *The Journal of Antimicrobial Chemotherapy, 66*(3), 531-538. doi:10.1093/jac/dkq496

Tiwari, P. B., Wang, X., He, J., & Darici, Y. (2015). Analyzing surface plasmon resonance data: Choosing a correct biphasic model for interpretation. *The Review of Scientific Instruments, 86*(3), 035001. doi:10.1063/1.4914027

Tiwari, P. B., Chapagain, P. P., Banda, S., Darici, Y., Üren, A., & Tse-Dinh, Y. (2016). Characterization of molecular interactions between escherichia coli RNA polymerase and topoisomerase I by molecular simulations. *FEBS Letters, 590*(17), 2844-2851. doi:10.1002/1873-3468.12321

Tiwari, P. B., Annamalai, T., Cheng, B., Narula, G., Wang, X., Tse-Dinh, Y. C., . . . Darici, Y. (2014). A surface plasmon resonance study of the intermolecular interaction between *Escherichia coli* topoisomerase I and pBAD/thio supercoiled plasmid DNA. *Biochemical and Biophysical Research Communications, 445*(2), 445-450. doi:10.1016/j.bbrc.2014.02.015 [doi]

Triccas, J. A., Parish, T., Britton, W. J., & Gicquel, B. (1998). An inducible expression system permitting the efficient purification of a recombinant antigen from *Mycobacterium smegmatis*. *FEMS Microbiology Letters, 167*(2), 151-156.

Trzcińska-Daneluti, A. M., Górecki, A., Czubaty, A., Kowalska-Loth, B., Girstun, A., Murawska, M., . . . Staroń, K. (2007). RRM proteins interacting with the cap region of topoisomerase I. *Journal of Molecular Biology, 369*(4), 1098-1112.

Tse-Dinh, Y. C. (1986). Uncoupling of the DNA breaking and rejoining steps of escherichia coli type I DNA topoisomerase. demonstration of an active covalent protein-DNA complex. *The Journal of Biological Chemistry, 261*(23), 10931-10935.

Tse-Dinh, Y. C. (1991). Zinc (II) coordination in *Escherichia coli* DNA topoisomerase I is required for cleavable complex formation with DNA. *The Journal of Biological Chemistry, 266*(22), 14317-14320.

Tse-Dinh, Y. C. (2007). Exploring DNA topoisomerases as targets of novel therapeutic agents in the treatment of infectious diseases. *Infectious Disorders Drug Targets, 7*(1), 3-9.

Tse-Dinh, Y. C., & Beran-Steed, R. K. (1988). *Escherichia coli* DNA topoisomerase I is a zinc metalloprotein with three repetitive zinc-binding domains. *The Journal of Biological Chemistry, 263*(31), 15857-15859.

Tse-Dinh, Y. (2009). Bacterial topoisomerase I as a target for discovery of antibacterial compounds. *Nucleic Acids Research, 37*(3), 731-737. doi:10.1093/nar/gkn936

Tse-Dinh, Y. (2015). Targeting bacterial topoisomerase I to meet the challenge of finding new antibiotics. *Future Medicinal Chemistry, 7*(4), 459-471. doi:10.4155/fmc.14.157

Uson, M. L., Ordonez, H., & Shuman, S. (2015). *Mycobacterium smegmatis* HelY is an RNA-activated ATPase/dATPase and 3'-to-5' helicase that unwinds 3'-tailed RNA duplexes and RNA:DNA hybrids. *Journal of Bacteriology, 197*(19), 3057-3065.

Van Die, I. M., Bergmans, H. E., & Hoekstra, W. P. (1983). Transformation in *Escherichia coli*: Studies on the role of the heat shock in induction of competence. *Journal of General Microbiology, 129*(3), 663-670.

Valenti, A., Perugino, G., Varriale, A., D'Auria, S., Rossi, M., & Ciaramella, M. (2010). The archaeal topoisomerase reverse gyrase is a helix-destabilizing protein that unwinds four-way DNA junctions. *The Journal of Biological Chemistry, 285*(47), 36532-36541. doi:10.1074/jbc.M110.169029 [doi]

Vassylyev, D. G. (2009). Elongation by RNA polymerase: A race through roadblocks. *Current Opinion in Structural Biology, 19*(6), 691-700.

Vassylyev, D. G., Vassylyeva, M. N., Perederina, A., Tahirov, T. H., & Artsimovitch, I. (2007). Structural basis for transcription elongation by bacterial RNA polymerase. *Nature, 448*(7150), 157-162. doi:10.1038/nature05932

Viard, T., & de la Tour, C B. (2007). Type IA topoisomerases: A simple puzzle? *Biochimie, 89*(4), 456-467. doi:10.1016/j.biochi.2006.10.013

Von Mering, C., Jensen, L. J., Snel, B., Hooper, S. D., Krupp, M., Foglierini, M., . . . Bork, P. (2005). STRING: Known and predicted protein–protein associations, integrated and transferred across organisms. *Nucleic Acids Research, 33* (Database Issue), D437. doi:10.1093/nar/gki005

Vos, S. M., Tretter, E. M., Schmidt, B. H., & Berger, J. M. (2011). All tangled up: How cells direct, manage and exploit topoisomerase function. *Nature Reviews Molecular Cell Biology, 12*(12), 827-841. doi:10.1038/nrm3228

Wall, M. E., & Wani, M. C. (1995). Camptothecin and taxol: Discovery to Clinic—Thirteenth bruce F. cain memorial award lecture. *Cancer Research, 55*(4), 753-760.

Wang, J. C. (1971). Interaction between DNA and an *Escherichia coli* protein omega. *Journal of Molecular Biology, 55*(3), 523-533.

Wang, J. (1996). DNA topoisomerases. *Annual Review of Biochemistry, 65*(1), 635-692. doi:10.1146/annurev.bi.65.070196.003223

Wang, J. C. (2002). Cellular roles of DNA topoisomerases: A molecular perspective. *Nature Reviews. Molecular Cell Biology, 3*(6), 430-440. doi:10.1038/nrm831

Wang, W., Yang, Y., Wang, S., Nagaraj, V. J., Liu, Q., Wu, J., & Tao, N. (2012). Label-free measuring and mapping of binding kinetics of membrane proteins in single living cells. *Nature Chemistry, 4*(10), 846-853. doi:10.1038/nchem.1434 [doi]

Wareed Ahmed, Claudia Sala, Shubhada R Hegde, Rajiv Kumar Jha, Stewart T Cole, & Valakunja Nagaraja. (2017). Transcription facilitated genome-wide recruitment of topoisomerase I and DNA gyrase. *PLoS Genetics, 13*(5), e1006754.

Watt, P. M., & Hickson, I. D. (1994). Structure and function of type II DNA topoisomerases. *The Biochemical Journal, 303 (Pt 3)*(Pt 3), 681-695.

Waugh, D. S. (2005). Making the most of affinity tags. *Trends in Biotechnology, 23*(6), 316-320. doi:10.1016/j.tibtech.2005.03.012

Willander, M., & Al-Hilli, S. (2009). Analysis of biomolecules using surface plasmons. *Methods in Molecular Biology (Clifton, N.J.), 544*, 201-229.

Williams, K. J., Joyce, G., & Robertson, B. D. (2010). Improved mycobacterial tetracycline inducible vectors. *Plasmid, 64*(2), 69-73.

Wilson, W. D. (2002). Tech.sight. Analyzing biomolecular interactions. *Science (New York, N.Y.), 295*(5562), 2103-2105. doi:10.1126/science.295.5562.2103 [doi]

Wohlkonig, A., Chan, P. F., Fosberry, A. P., Homes, P., Huang, J., Kranz, M., . . . Bax, B. D. (2010). Structural basis of quinolone inhibition of type IIA topoisomerases and target-mediated resistance. *Nature Structural & Molecular Biology, 17*(9), 1152-1153. doi:10.1038/nsmb.1892

Wu, C., Li, T., Farh, L., Lin, L., Lin, T., Yu, Y., . . . Chan, N. (2011). Structural basis of type II topoisomerase inhibition by the anticancer drug etoposide. *Science (New York, N.Y.), 333*(6041), 459-462. doi:10.1126/science.1204117

Wu, Y., & Datta, P. (1995). Influence of DNA topology on expression of the tdc operon in *Escherichia coli* K-12. *Molecular & General Genetics: MGG, 247*(6), 764-767.

Wu, H. Y., Shyy, S. H., Wang, J. C., & Liu, L. F. (1988). Transcription generates positively and negatively supercoiled domains in the template. *Cell, 53*(3), 433-440.

Wu, L., Chan, K. L., Ralf, C., Bernstein, D. A., Garcia, P. L., Bohr, V. A., . . . Hickson, I. D. (2005). The HRDC domain of BLM is required for the dissolution of double holliday junctions. *The EMBO Journal, 24*(14), 2679-2687. doi:7600740 [pii]

Wu, L., Davies, S. L., North, P. S., Goulaouic, H., Riou, J. F., Turley, H., . . . Hickson, I. D. (2000). The bloom's syndrome gene product interacts with topoisomerase III. *The Journal of Biological Chemistry, 275*(13), 9636-9644.

Wu, L., & Hickson, I. D. (2003). The bloom's syndrome helicase suppresses crossing over during homologous recombination. *Nature, 426*(6968), 870-874.

Wuchty, S., & Uetz, P. (2014). Protein-protein interaction networks of *E. coli* and *S. cerevisiae* are similar. *Scientific Reports, 4*, 7187. doi:10.1038/srep07187 [doi]

Xu, D., Tsai, C. J., & Nussinov, R. (1997). Hydrogen bonds and salt bridges across protein-protein interfaces. *Protein Engineering, Design and Selection, 10*(9), 999-1012. doi:10.1093/protein/10.9.999

Xu, W., Zhang, L., Mai, J., Peng, R., Yang, E., Peng, C., & Wang, H. (2014). The Wag31 protein interacts with AccA3 and coordinates cell wall lipid permeability and lipophilic drug resistance in *Mycobacterium smegmatis*. *Biochemical and Biophysical Research Communications, 448*(3), 255-260.

Xu, D., Shen, W., Guo, R., Xue, Y., Peng, W., Sima, J., . . . Wang, W. (2013). Top3β is an RNA topoisomerase that works with fragile X syndrome protein to promote synapse formation. *Nature Neuroscience, 16*(9), 1238.

Xue, L. C., Dobbs, D., Bonvin, Alexandre M J J, & Honavar, V. (2015). Computational prediction of protein interfaces: A review of data driven methods. *FEBS Letters, 589*(23), 3516-3526.

Yakovlev, A. A. (2009). Crosslinkers and their utilization for studies of intermolecular interactions. *Neurochemical Journal, 3*(2), 139-144.

Yan, C., Wu, F., Jernigan, R. L., Dobbs, D., & Honavar, V. (2008). Characterization of protein-protein interfaces. *The Protein Journal, 27*(1), 59-70.

Yang, Q., Huang, F., Hu, L., & He, Z. G. (2012). Physical and functional interactions between 3-methyladenine DNA glycosylase and topoisomerase I in mycobacteria. *Biochemistry, 77*(4), 378-387.

Yang, J., Annamalai, T., Cheng, B., Banda, S., Tyagi, R., & Tse-Dinh, Y. C. (2015). Antimicrobial susceptibility and SOS-dependent increase in mutation frequency are impacted by *Escherichia coli* topoisomerase I C-terminal point mutation. *Antimicrobial Agents and Chemotherapy, 59*(10), 6195-6202.

Yang, Q., Liu, Y., Huang, F., & He, Z. G. (2011). Physical and functional interaction between D-ribokinase and topoisomerase I has opposite effects on their respective activity in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. *Archives of Biochemistry and Biophysics, 512*(2), 135-142. doi:10.1016/j.abb.2011.05.018 [doi]

Yang, X., Li, W., Prescott, E. D., Burden, S. J., & Wang, J. C. (2000). DNA topoisomerase II beta and neural development. *Science (New York, N.Y.), 287*(5450), 131-134.

Yang, Y., McBride, K. M., Hensley, S., Lu, Y., Chedin, F., & Bedford, M. T. (2014). Arginine methylation facilitates the recruitment of TOP3B to chromatin to prevent R loop accumulation. *Molecular Cell, 53*(3), 484-497.

Yu, C. E., Oshima, J., Fu, Y. H., Wijsman, E. M., Hisama, F., Alisch, R., . . . Schellenberg, G. D. (1996). Positional cloning of the werner's syndrome gene. *Science (New York, N.Y.), 272*(5259), 258-262.

Zhang, H. L., & DiGate, R. J. (1994). The carboxyl-terminal residues of *Escherichia coli* DNA topoisomerase III are involved in substrate binding. *Journal of Biological Chemistry, 269*(12), 9052-9059.

Zhang, H. L., Malpure, S., Li, Z., Hiasa, H., & DiGate, R. J. (1996). The role of the carboxyl-terminal amino acid residues in *Escherichia coli* DNA topoisomerase III-mediated catalysis. *Journal of Biological Chemistry, 271*(15), 9039-9045.

Zhang, Y., Regairaz, M., Seiler, J. A., Agama, K. K., Doroshow, J. H., & Pommier, Y. (2011). Poly(ADP-ribose) polymerase and XPF-ERCC1 participate in distinct pathways for the repair of topoisomerase I-induced DNA damage in mammalian cells. *Nucleic Acids Research, 39*(9), 3607-3620. doi:10.1093/nar/gkq1304

Zhu, C. X., Qi, H. Y., & Tse-Dinh, Y. C. (1995). Mutation in Cys662 of *Escherichia coli* DNA topoisomerase I confers temperature sensitivity and change in DNA cleavage selectivity. *Journal of Molecular Biology, 250*(5), 609-616.

Zhu, C. X., & Tse-Dinh, Y. C. (1999). Overexpression and purification of bacterial DNA topoisomerase I. *Methods in Molecular Biology (Clifton, N.J.), 94*, 145-151. doi:145

VITA

SRIKANTH BANDA

2012-2017	PhD., Biochemistry Florida International University Miami, FL, USA
2008-2010	M.S., Analytical Chemistry Governors State University University park, IL, USA
2003-2007	Bachelor of Pharmacy Kakathiya University, Warangal, India

PUBLICATIONS AND PRESENTATIONS

Banda et al., Novel mechanism evolved for Mycobacteria RNA polymerase and Topoisomerase I Protein-Protein Interaction. Manuscript will be submitted to Journal of Molecular Biology.

Tiwari, Banda et al., Exploring Molecular Interactions between *Escherichia coli* RNA Polymerase and Topoisomerase I by Molecular Simulations, Biophysical journal, 112 (3), p287a, Feb 2017.

Tiwari, Banda et al., Characterization of molecular interactions between *E. coli* RNA polymerase and Topoisomerase I by molecular simulations. FEBS Letters, 590(17), 2844-51, September 2016

Banda et al., Investigating Direct Protein interactions of Topoisomerase I and RecA in *E. coli*. Gene (585), 65-70, March 2016.

Yang, Banda et al., Antimicrobial susceptibility and SOS-dependent increase in mutation frequency are impacted by *E. coli* Topoisomerase I C-terminal point mutation. Antimicrobial Agents and Chemotherapy; 59 (10), 6195-202, Oct 2015.

Banda et al., Cis-Platin Photo-Analogs, $Pt(dppz)Cl_2$ and $Pt(bpy)Cl_2$, as Potential Photodynamic Therapeutic Agents. Conference paper, 38th American Chemical Society Great Lakes Regional Meeting, May 2009.

Protein-protein interactions of bacterial topoisomerase I, Poster presentation, Gordon Research Conference, Newry, ME; August 2016.

Protein interactions of Mycobacterial topoisomerase I, Poster presentation, American Chemical Society Meeting, Florida Section, May 2015.

Protein interactions of Mycobacterial topoisomerase I, Presentation, Graduate Student Appreciation Week, Florida International University, April 2015.

Protein interactions of Mycobacterial topoisomerase I, Poster presentation, Gordon Research Conference, Newry, ME; August 2014.

Ruthenium complexes as potential photodynamic agents, Presentation, 42nd Central Regional Meeting of the ACS, Dayton, Ohio; May 2009.

Dirhodium complexes as potential photodynamic anticancer agents, Presentation, Student Research Symposium, Governors State University, May 2009.

Strategies to deliver drugs across blood brain barrier, Poster presentation, Student Research Symposium, Bheemavaram, India, November 2006.