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Understanding Substrate Selection and Functional Output of Bacterial Type IIA
Topoisomerase Orthologs and Paralogs

By

Elsa Marie Tretter

A dissertation submitted in satisfaction of the
requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor James M. Berger, Chair

Professor Tom Alber

Professor Jan Liphardt

Professor Andreas Martin

Spring 2012

Abstract

Understanding Substrate Selection and Functional Output of Bacterial Type IIA Topoisomerase Orthologs and Paralogs

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Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor James M. Berger, Chair

Key processes in the cell, such as replication, transcription and repair, result in the supercoiling of genomic DNA, which is resolved by molecular machines known as topoisomerases. Type II DNA topoisomerases remodel DNA by passing one duplex DNA strand through a transient break in a second duplex DNA strand. Type II topoisomerases achieve DNA cleavage and religation with high fidelity by the coordination of multiple domains. Achilles heel-like enzymes, which are indispensable to cells, topoisomerases are targeted by numerous antibiotics and cancer therapeutics, necessitating a thorough understanding of their structure and function.

Previously thought to be a uniform group of enzymes, a number of bacterial type IIA topoisomerase paralogs have been identified with highly specialized functions but it is not understood how each paralog recognizes and acts on unique DNA topologies. The central hypothesis of this project is that the bacterial type IIA DNA binding domain, termed the C-terminal domain (CTD), is principally responsible for substrate identification and functional output of type IIA topoisomerases. I performed an array of structural and biochemical studies on three unique bacterial type IIA topoisomerases to understand how the CTD controls type IIA topoisomerase activity. The work presented in this dissertation identifies novel type IIA topoisomerase evolutionary paths, characterizes novel regulatory mechanisms, and introduces evidence that many characteristics refine enzyme activity.

Table of Contents

Chapter 1 – Introduction to DNA Topoisomerases.....	1
The Role of DNA Topoisomerases/ DNA Topology.....	1
Variety and Classification of DNA Topoisomerases.....	1
Structure and Mechanism of Type IIA Topoisomerases.....	3
Specialization of Bacterial Type IIA Topoisomerase Paralogs.....	4
Tables	
Table 1.1. Overview of topoisomerase class characteristics.....	6
Figures	
Figure 1.1. Global DNA winding and entanglement resolved by topoisomerases.....	7
Figure 1.2. Illustrations of topoisomerase mechanisms by class.....	8
Figure 1.3. Ribbon models and sequence alignments of representative CTD.....	9
Chapter 2 – Crystallization of the <i>M. tuberculosis</i> Gyrase N-terminal	
Domain.....	10
Introduction.....	10
Results and Discussion	10
Materials and Methods.....	11
Protein Purification.....	11
Crystallization.....	11
X-ray diffraction data collection and structure determination.....	12
Tables	
Table 2.1. Data collection and refinement statistics.....	13
Figures	
Figure 2.1. <i>MtGyr59</i> structure.....	14
Chapter 3 – Mechanisms for defining the supercoiling setpoint of DNA	
gyrase orthologs. Part I. A non-conserved acidic C-terminal tail modulates	
<i>E. coli</i> gyrase activity.....	15
Introduction.....	15
Results and Discussion.....	16
Control of DNA wrapping by the negatively-charged <i>E. coli</i> tail.....	16

Regulation of DNA binding by the GyrA tail.....	17
Effects of tail on DNA wrapping by the gyrase holoenzyme.....	17
Impact of the GyrA tail on DNA supercoiling by gyrase.....	18
Effect of the tail on gyrase ATPase activity.....	19
Conclusion.....	19
Materials and Methods.....	21
Protein purification.....	21
Topological footprinting assays.....	21
DNA binding assays.....	22
DNA relaxation and supercoiling assays.....	22
ATPase assays.....	23
Figures	
Figure 3.1. Bacterial type IIA topoisomerase structure and tail alignment.....	24
Figure 3.2. GyrA and CTD topology footprint assays.....	25
Figure 3.3. DNA binding by CTD constructs.....	26
Figure 3.4. Gyrase holoenzyme topology footprint assays.....	27
Figure 3.5. Comparison of supercoiling extent by wild-type and insert-less <i>E. coli</i> gyrase.....	28
Figure 3.6. ATP-independent, negative supercoil relaxation by wild-type and insert-less <i>E. coli</i> gyrase holoenzyme.....	29
Figure 3.7. ATP hydrolysis by wild-type and insert-less <i>E. coli</i> gyrase.....	30
Figure 3.8. Model accounting for the action of the CTD tail on <i>E. coli</i> gyrase.....	31
Supplemental Material.....	33
Figure S3.1. SDS-PAGE gels of purified <i>E. coli</i> gyrase proteins.....	33
Chapter 4 – Mechanisms for defining the supercoiling setpoint of DNA gyrase orthologs. Part II. The shape of the GyrA CTD is not a sole determinant for controlling supercoiling efficiency	34
Introduction.....	34
Results and Discussion.....	34
Structure of the <i>Mtb</i> GyrA CTD.....	34

DNA binding and wrapping by the <i>Mtb</i> CTD and <i>Mtb</i> gyrase.....	35
Decatenation by <i>Mtb</i> gyrase.....	36
DNA supercoiling by <i>Mtb</i> gyrase.....	36
ATPase activity of <i>Mtb</i> gyrase.....	37
Conclusion.....	38
Materials and Methods.....	39
Protein purification.....	39
Crystallization, data collection, and structure solution.....	40
DNA binding assays.....	40
DNA relaxation, decatenation, and supercoiling assays.....	41
Topological footprinting assays.....	41
ATPase assays.....	42
Tables	
Table 4.1. Data collection and refinement statistics.....	43
Figures	
Figure 4.1. <i>M. tuberculosis</i> CTD structure.....	44
Figure 4.2. DNA binding by CTD constructs.....	45
Figure 4.3. Topology footprint assays.....	46
Figure 4.4. <i>E. coli</i> and <i>M. tuberculosis</i> holoenzyme decatenation assay.....	47
Figure 4.5. <i>E. coli</i> and <i>M. tuberculosis</i> negative supercoiling holoenzyme assays....	48
Figure 4.6. Negative supercoiling time course assay using 5 nM and 20 nM holoenzyme.....	49
Figure 4.7. ATPase activity of <i>E. coli</i> and <i>M. tuberculosis</i> gyrase.....	50
Figure 4.8. Schematic comparing the relative supercoiling setpoints of a select number of bacterial type IIA topoisomerases.....	51
Supplemental Material.....	52
Figure S4.1. SDS-PAGE gels of purified <i>Mtb</i> and <i>E. coli</i> gyrase proteins.....	52
Figure S4.2. Wild-type and Insert-less <i>E. coli</i> holoenzyme decatenation.....	53
Chapter 5 – Evolution and Specialization of the <i>A. aeolicus</i> type IIA DNA Topoisomerase.....	54

Introduction.....	54
Results and Discussion.....	55
Structure of GyrA C-terminal domain.....	55
<i>Aquifex aeolicus</i> holoenzyme activity.....	56
DNA writhing introduction by the <i>A. aeolicus</i> ParC C-terminal domain.....	57
The type II topoisomerase of <i>A. aeolicus</i> is a gyrase/topo IV hybrid.....	57
<i>A. aeolicus</i> GyrB supports negative supercoiling activity.....	57
<i>A. aeolicus</i> : <i>T. maritima</i> GyrA fusion supports negative supercoiling activity.....	58
Conclusion.....	58
Materials and Methods.....	60
Protein purification.....	60
Crystallization, data collection, and structure solution.....	60
DNA relaxation, decatenation, and supercoiling assays.....	61
Topological footprinting assays.....	61
Sequence alignments and phylogenetic trees.....	62
Updated genome annotation and ParC gene length for <i>A. aeolicus</i> topo IV.....	62
Tables	
Table 5.1. Data collection and refinement statistics.....	63
Table 5.2. Sequences used in MAFFT sequence alignments.....	64
Figures	
Figure 5.1. Type IIA topoisomerase functions and structure.....	67
Figure 5.2. <i>A. aeolicus</i> CTD structure.....	68
Figure 5.3. <i>A. aeolicus</i> holoenzyme assays.....	69
Figure 5.4. Negative supercoiling chimera activities.....	70
Figure 5.5 Model for bacterial type IIA DNA topoisomerase evolution and Specialization.....	71
Supplemental Material.....	72
Figure S5.1. Sequence alignment between representative CTDs.....	72
Figure S5.2. C-terminal domain surface charges and alignment statistics.....	73
Figure S5.3. Topoisomerase DNA wrapping and activity assays.....	74

Figure S5.4. Phylogenetic trees comparing sequences.....	75
Figure S5.5. Phylogenetic tree comparing sequences for GyrB/ParE.....	76
Figure S5.6. SDS-PAGE gels of purified <i>A. aeolicus</i> proteins.....	77
Chapter 6 – Conclusions and Future Directions.....	78
Conclusions.....	78
Future Directions.....	79
References.....	80
Appendix.....	87
Auto-Induction Protein Expression.....	87
Large-scale <i>in-vivo</i> recombination to generate minicircle DN.....	90
DNA Minicircle Purification.....	96
kDNA Purification.....	100
Chloroquine Gels.....	102
Topoisomerase Writhe Assay.....	103
<i>M. tuberculosis</i> Gyrase Expression and Purification.....	105

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Chapter 1 – Introduction to DNA Topoisomerases

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The Role of DNA Topoisomerases/ DNA Topology

The structure of deoxyribonucleic acid (DNA) provides a protective and stable genetic information storage system, but it also presents a number of challenges to the cell. Since the strands of duplex DNA are wound around each other, the information encoded by the DNA bases is hidden within the helix. Processes such as replication and transcription require local unwinding of the double helix, which results in global DNA overwinding and entanglement (Liu and Wang 1987). Specifically, compensatory positive supercoils, DNA twisted more than 10.5 base pairs per turn, are formed ahead of replication and transcription forks, whereas negative supercoils, DNA twisted less than 10.5 base pairs per turn, form behind the transcription fork and precatenae form behind the replication fork (**Figure 1.1**) (Wu, Shyy et al. 1988). Failure to properly regulate DNA topology can result in chromosome missegregation, DNA breakage, and stalled transcription and replication forks (Wu, Shyy et al. 1988).

To overcome these topological challenges, cells utilize a class of enzymes known as DNA topoisomerases. DNA topoisomerases resolve topological problems by cleaving one or both strands of the DNA double helix, which results in negative supercoil introduction, relaxation of positive and negative supercoils, or DNA decatenation (Schoeffler and Berger 2008). DNA topoisomerases are ubiquitous, essential enzymes responsible for regulating DNA topology.

Variety and Classification of DNA Topoisomerases

Cells have evolved distinct types of topoisomerases that modify and manage DNA topology using single- or double-strand DNA breaks. DNA strand cleavage is the main topoisomerase classification scheme that divides topoisomerases into two main groups, type I and type II. Type I enzymes cleave a DNA single-strand and type II enzymes cleave both DNA strands. The two types are currently subdivided into five subfamilies: IA, IB, IC, IIA, and IIB. A comparison of these different subclasses of topoisomerases is summarized in **Table 1.1** and **Figure 1.2**. Regardless of topoisomerase type or subfamily, all topoisomerases utilize a catalytic tyrosine to attack the phosphodiester backbone of DNA, which generates a transient, DNA-topoisomerase covalent phosphotyrosine linkage that protects the DNA ends during enzyme turnover and prevents DNA nicks and breaks (**Figure 1.2A**). If mismanaged, cleaved DNA-topoisomerase complexes can create potentially lethal DNA strand breaks.

Type IA enzymes separate short stretches of double-stranded (ds) DNA making a transient, single-strand break which creates an 5'-phosphotyrosyl DNA:enzyme bridge that is re-sealed on the opposite side of the intact DNA strand (Depew, Liu et al. 1978; Liu and Wang 1979; Tse, Kirkegaard et al. 1980) (**Figure 1.2B**). Additionally, type IA topoisomerases require Mg^{2+} and Zn^{2+} for full activity (Tse-Dinh and Beran-Steed 1988; Domanico and Tse-Dinh 1991). Type IA DNA topoisomerases can decatenate or pass one duplex DNA strand through another if one of the DNA strands already contains a nick or

gap (Brown and Cozzarelli 1981). Within the Type IA enzymes, topo I preferentially relaxes negative supercoils because it is partially unwound but it can also relax positively supercoiled DNA if a single-stranded stretch of DNA exists (Kirkegaard and Wang 1985). Conversely, topo III resolves complicated single-stranded DNA structures that result from DNA replication and recombination (Srivenugopal, Lockshon et al. 1984; Kirkegaard and Wang 1985; Wallis, Chrebet et al. 1989). A unique member of the type IA family, reverse gyrase is found in thermophilic bacteria and archaea and resembles topo I and III in fold and structure; however, it also contains a helicase-like domain that uses ATP to actively introduce positive supercoils into DNA, which is thought to combat thermal denaturation (Kikuchi and Asai 1984).

Type IB enzymes are specific to eukaryotes and viruses although homologs exist in bacteria (Krogh and Shuman 2002). After forming a 3'-phosphotyrosyl linkage to DNA, type IB enzymes utilize a metal-independent swivelase or rotation mechanism where the energy contained in supercoiled DNA is carefully exploited to rotate the free end of one transiently cleaved DNA strand around another fixed DNA strand (Champoux 1981; Taneja, Schnurr et al. 2007) (**Figure 1.2C**). This mechanism permits type IB enzymes to relax positively and negatively supercoiled DNA with equal affinity (Koster, Croquette et al. 2005). The number of DNA relaxation revolutions per strand cleavage/re-ligation event is proportional to the density of supercoils originally present in the DNA.

Type IC enzymes are, to date, only found in archaea and are functionally similar to topo IB enzymes in that they create a 3'-phosphotyrosyl linkage to DNA, relax positive and negatively supercoiled DNA, and do not require metals or ATP (Slesarev, Stetter et al. 1993; Forterre 2006; Taneja, Schnurr et al. 2007) (**Figure 1.2C**). They are unique, however, because they repair DNA by removing abasic lesions using apurinic site (AP) lyase activity (Belova, Prasad et al. 2001).

Type II enzymes employ a similar protein-mediated strand passage mechanism as type IA enzymes; however, type II enzymes cleave one dsDNA strand and pass a second, intact dsDNA strand through the transient break (Brown and Cozzarelli 1979; Liu, Liu et al. 1980; Mizuuchi, Fisher et al. 1980) (**Figure 1.2D**). Strand passage is controlled by the navigation of DNA through several protein 'gates,' which are controlled by ATP hydrolysis (Mizuuchi, Fisher et al. 1980; Roca and Wang 1992). This strand passage mechanism regulates and drives a distinct, unidirectional strand passage event resulting in decatenation, supercoil relaxation, or negative supercoil addition.

Different Type II enzymes have evolved preferences for specific DNA substrates. Bacterial topo IV and eukaryotic topo II both decatenate DNA strands and relax supercoils (Peng and Marians 1993; Crisona, Strick et al. 2000). Higher eukaryotes possess two topo II isoforms named topo II α / II β and the reason for the existence of two isoforms is not fully understood but recent studies have showed that topo II α is expressed constitutively while topo II β is associated with DNA repair and development (Drake, Zimmerman et al. 1987; Drake, Hofmann et al. 1989; Forterre, Gribaldo et al. 2007). Organisms can utilize different combinations of topoisomerases, enzyme paralogs, or enzyme isoforms to maintain their chromosome topology (Forterre, Gribaldo et al. 2007).

DNA Gyrase is unique in that it actively adds negative supercoils to DNA and is thought to help maintain the negative supercoiled state of bacterial genomes (Zechiedrich, Khodursky et al. 2000). It is believed that DNA gyrase was the original type IIA bacterial topoisomerase and topo IV was formed via gene duplication and

subsequently lost its supercoiling activity and alternatively specialized in supercoil relaxation and decatenation (Forterre, Gribaldo et al. 2007). This example illustrates one of many evolutionary paths topoisomerases have traveled to adapt to meet specific cellular needs.

Type IIB topoisomerases are found in the archaea, plants, and a few bacterial, protist, and algal lineages (Gadelle, Filée et al. 2003). As with their type IIA cousins, type IIB topoisomerases unlink tangled DNA duplexes by strand passage, and relax both negative and positive supercoils (Bergerat, Gadelle et al. 1994). Type IIB topoisomerases also possess ATPase and DNA-cleavage domains similar to those found in type IIA enzymes, although the relative arrangement of these elements in primary sequence space and their overall structural organization differs significantly (**Figure 1.2E**) (Bergerat, Gadelle et al. 1994; Bergerat, de Massy et al. 1997; Nichols, DeAngelis et al. 1999; Corbett and Berger 2003). The principal DNA binding subunit of type IIB topoisomerases is noteworthy in that it is evolutionarily related to Spo11, the factor responsible for creating double-stranded DNA breaks that initiate meiotic recombination (Bergerat, de Massy et al. 1997; Keeney, Giroux et al. 1997). Thus far, only one family member, topo VI, is known to comprise the type IIB topoisomerase clade (Bergerat, de Massy et al. 1997).

Structure and Mechanism of Type IIA Topoisomerases

Type IIA DNA topoisomerases manage DNA topology by a “strand passage” mechanism, whereby one intact DNA duplex is physically passed through a transient break in another DNA duplex (**Figure 1.2D**). There are two type IIA topoisomerases paralogs in bacteria, gyrase (encoded by *gyrA* and *gyrB*) and topoIV (encoded by *parC* and *parE*), both of which are heterotetramers (Sugino, Higgins et al. 1980). DNA strand passage begins when one DNA duplex, termed the Gate or G segment, binds to the DNA binding and cleavage interface called the DNA gate (Roca and Wang 1992; Berger, Gamblin et al. 1996; Morais Cabral, Jackson et al. 1997). The DNA-gate is composed of a metal binding TOPRIM domain and a winged helix domain (WHD). The WHD contains the catalytic tyrosine that forms a covalent link to the DNA backbone via a phosphotyrosine linkage (Berger, Gamblin et al. 1996).

Upon binding ATP, the GHKL ATPase domains dimerize and capture a second DNA duplex called the Transfer or T segment (Wigley, Davies et al. 1991; Roca and Wang 1992; Ali, Jackson et al. 1993). Changes in gate conformation that accompany ATP hydrolysis and phosphate release allow passage of the T segment through the cleaved G segment and out the open coiled-coil domains that form the Exit or C-gate (Berger, Gamblin et al. 1996; Roca, Berger et al. 1996). The enzyme is reset once the hydrolysis products are released and the ATPase domains reopen (Wigley, Davies et al. 1991; Roca and Wang 1992). Type IIA topoisomerases unidirectionally transport one DNA duplex through another and shift the DNA topoisomer distribution by acting on specific DNA substrates.

Specialization of Bacterial Type IIA Topoisomerase Paralogs

Most bacteria produce two closely related type IIA topoisomerase paralogs, DNA gyrase and topo IV (Kato, Nishimura et al. 1990). Topo IV relaxes both positive and negative supercoils and is a robust decatenase that separates daughter chromosomes and precatenates during DNA replication (Zechiedrich, Khodursky et al. 1997; Crisona, Strick et al. 2000). In contrast, DNA gyrase introduces negative supercoils into DNA to keep bacterial chromosomes in a negatively-supercoiled state, which encourages compaction and unwinding, and is considered a poor decatenase (Gellert, Mizuuchi et al. 1976; Zechiedrich, Khodursky et al. 2000).

To achieve unique functional output despite high sequence and structure similarity, gyrase and topo IV modify how the DNA binding domain appended to the enzyme core, called the C-terminal domain (CTD) interacts with DNA. It is currently believed that topo IV interacts with two DNA strands, in *trans*, which increases the fidelity for its preferred substrates (Peng and Mariani 1995; Crisona, Strick et al. 2000; Stone, Bryant et al. 2003). Conversely, DNA gyrase wraps one DNA duplex around the enzyme's exterior and performs strand passage in *cis*, allowing a negative supercoil to be introduced upon strand passage (Brown and Cozzarelli 1979; Corbett, Shultzaberger et al. 2004).

The CTD consists of several duplicated β -sheets arranged in a " β -pinwheel" configuration similar to, but topologically distinct from, a β -propeller (Reece and Maxwell 1991; Corbett, Shultzaberger et al. 2004). Several structures of CTDs have been solved and they display a wide range of structural diversity (**Figure 1.3**). Some CTDs adopt an 'open' or 'C' shaped ring versus a 'closed' ring. Additionally, some CTDs are planar pinwheel shapes and others are in a bent, 'lock-washer' configuration. Gyrase orthologs are known to only possess six-bladed CTDs, whereas topo IV CTDs can vary greatly and are thought to have anywhere from three to seven blades. Perhaps the DNA gyrase CTD is under stringent selective pressure and requires six DNA wrapping blades for enzyme activity, while the topo IV CTD is free to evolve for other purposes. Currently it is unclear how the structural variety of the CTD relates or contributes to its holoenzyme's functional output.

Regardless of its variation between type IIA topoisomerase orthologs and paralogs, the CTD is critical for enzyme activity. Removal of the gyrase CTD destroys gyrase's ability to negatively supercoil DNA, but not its ability to decatenate DNA in the absence of ATP (Kampranis and Maxwell 1996). Similarly, topo IV's preferential decatenation and relaxation of positive supercoils is lost when its CTD is removed (Corbett, Schoeffler et al. 2005).

A highly conserved motif found only in Gyrase, termed the GyrA Box, (QRRGGKG consensus) lies in the first blade (Ward and Newton 1997). Degenerate GyrA box sequences can also be found in many of the remaining blades, which suggests the CTD blades were formed through duplication events (Schoeffler and Berger 2008). By comparing the DNA wrapping and supercoiling activities of wild-type and mutant gyrase, it was shown the GyrA box motif is required for the negative supercoiling activity of the enzyme but the mutant still possessed wild type relaxation and decatenation activity (Kramlinger and Hiasa 2006). It is thought that the gyrase CTD bends DNA by inserting the GyrA box arginine residues into DNA's minor groove and creating kinks

that are comparable to the DNA wrapped nucleosome (Schoeffler and Berger 2008). Footprinting experiments that support this model revealed three distinct kinking sites where the CTD may bind (Oram, Travers et al. 2006).

Phylogenetic studies suggest that DNA gyrase was the original type IIA bacterial topoisomerase, and its paralog, topo IV, was formed via gene duplication (Forterre and Gabelle 2009). Topo IV is thought to have subsequently lost its supercoiling activity and alternatively specialized in supercoil relaxation and decatenation. This example illustrates one of many evolutionary paths topoisomerases have traveled to evolve to meet unique topological requirements in different organisms.

In this dissertation, I examine how the type IIA topoisomerase C-terminal DNA binding domain, found in DNA gyrase and topo IV, fine-tunes the enzyme's substrate specificity and modulates its functional output in three specific instances. I also describe the crystallographic structure of the *M. tuberculosis* gyrase N-terminal domain and discuss its differences and unique characteristics when compared to the previously solved *E. coli* gyrase N-terminal domain structure (**Chapter 2**).

An in-depth biochemical characterization of the *E. coli* gyrase CTD uncovered an unconserved, acidic tail at the extreme C-terminus that regulates the enzyme's interaction with DNA and increases the coupling efficiency between ATP turnover and supercoiling. Thus, intrinsic properties of gyrase, such as DNA binding and wrapping, can be fine-tuned by CTD modifications (**Chapter 3**).

Structural and biochemical comparison of *E. coli* and *Mycobacterium tuberculosis* gyrases revealed that CTD structure is not the sole regulatory determinant of gyrase activity. Additional characteristics, such as diminished ATPase activity and inability to couple DNA wrapping and supercoiling, play a key role. This indicated that gyrase was modified in multiple ways throughout evolution to regulate its specific catalytic properties (**Chapter 4**).

A structural and biochemical investigation of the sole type II topoisomerase from *Aquifex aeolicus*, a thermophile, revealed that it is the first bacterium found to lack gyrase and encode only topoIV. This enzyme shows an unusual modularity, whereby the CTD only is related to topoIV, while the rest of the enzyme closely resembles gyrase. I discovered a novel evolutionary path for the emergence of functional topoisomerase paralogs where a gyrase enzyme is directly converted to topoIV without gene duplication. Subsequently, I was able to convert a topoIV into a gyrase that supercoiled DNA by appending a thermophilic gyrase CTD to the *A. aeolicus* enzyme core (**Chapter 5**).

Prior to these efforts, little was known about how type II topoisomerases evolved multiple, species-specific modes of regulation to fine-tune and manage their function. My work highlights that the CTD is highly adaptable, modifying topoisomerase function through species-specific modifications and novel regulatory elements.

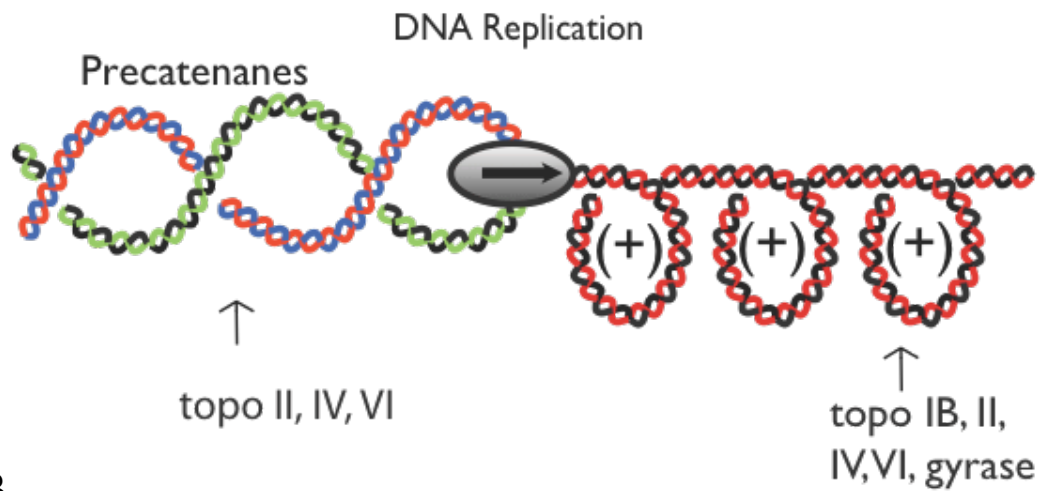
Tables

Family	Representatives	DNA Cleavage Mechanism	ATP dependent?	Metal dependent?
Type IA	Bacterial topo I & III Yeast topo III Mammalian topo III α & III β Archaeal Reverse Gyrase*	Single Strand Bridge, 5' phosphotyrosyl bond	No / Yes*	Yes
Type IB	Eukaryotic topo I	Single Strand Swivel, 3' phosphotyrosyl bond	No	No
Type IC	Archaeal <i>Methanopyrus</i> genus	Single Strand Swivel, 3' phosphotyrosyl bond	No	No
Type IIA/B	Bacterial DNA gyrase & topo IV Yeast topo II Mammalian topo II α & II β	Double Strand Bridge, 5' phosphotyrosyl bond	Yes	Yes

Table 1.1. Overview of topoisomerase class characteristics.

Figures

A



B

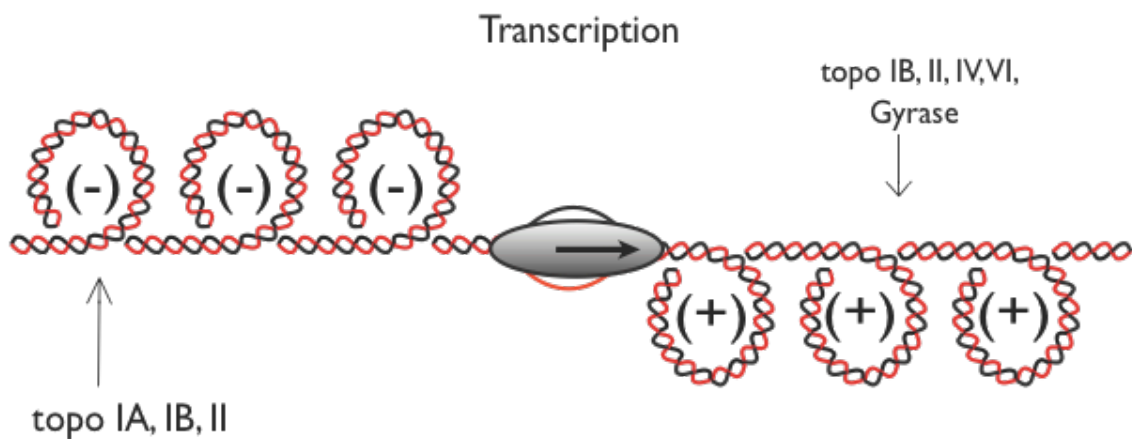


Figure 1.1. Global DNA winding and entanglement resolved by topoisomerases. A) As a replication fork progresses (grey sphere), positive supercoils form ahead of the fork, which are removed by topoisomerase IB, II, IV, VI, and DNA gyrase, while newly-replicated precatenanes form behind it and are removed by topoisomerase II, IV, and VI. B) As transcription progresses, positive supercoils form ahead of the moving polymerase (grey sphere) and are removed by topoisomerase IB, II, IV, VI, and DNA gyrase. Negative supercoils form behind the polymerase (grey sphere) and are removed by topoisomerase IA, IB, and II.

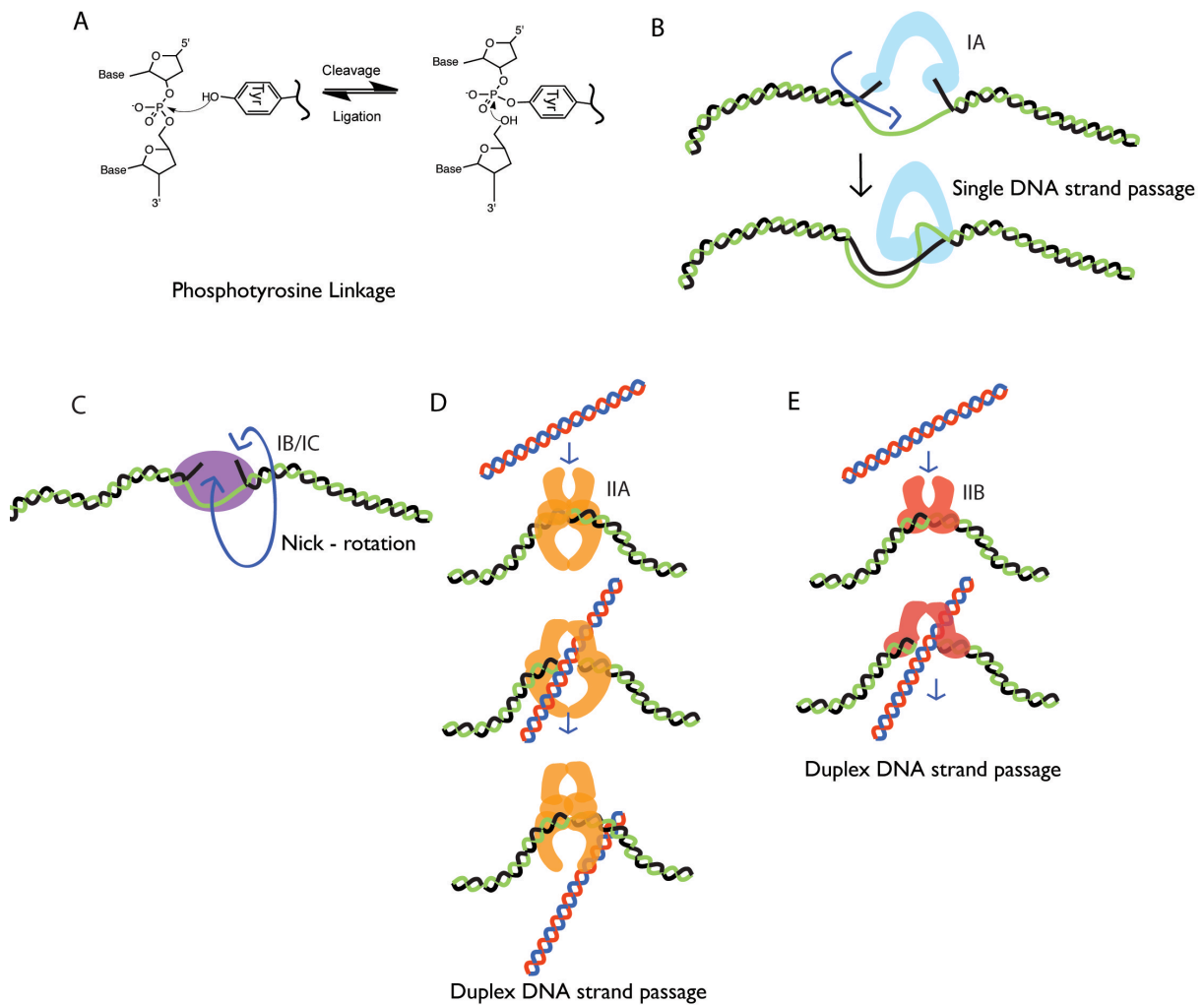


Figure 1.2. Illustrations of topoisomerase mechanisms by class. A) Transient cleavage of DNA by topoisomerases. A reversible covalent topoisomerase-DNA intermediate is formed when transesterification between an enzyme tyrosine and a DNA phosphate group breaks the DNA backbone bond. The DNA is re-annealed when the reaction is reversed (reaction shown depicts a 5' enzyme-DNA linkage, which is applicable to all topoisomerase families except topo IB which forms a 3' enzyme-DNA linkage). B) Type IA topoisomerase strand passage mechanism. C) Type IB/IC rotary mechanism. D) Type IIA strand passage mechanism. E) Type IIB strand passage mechanism

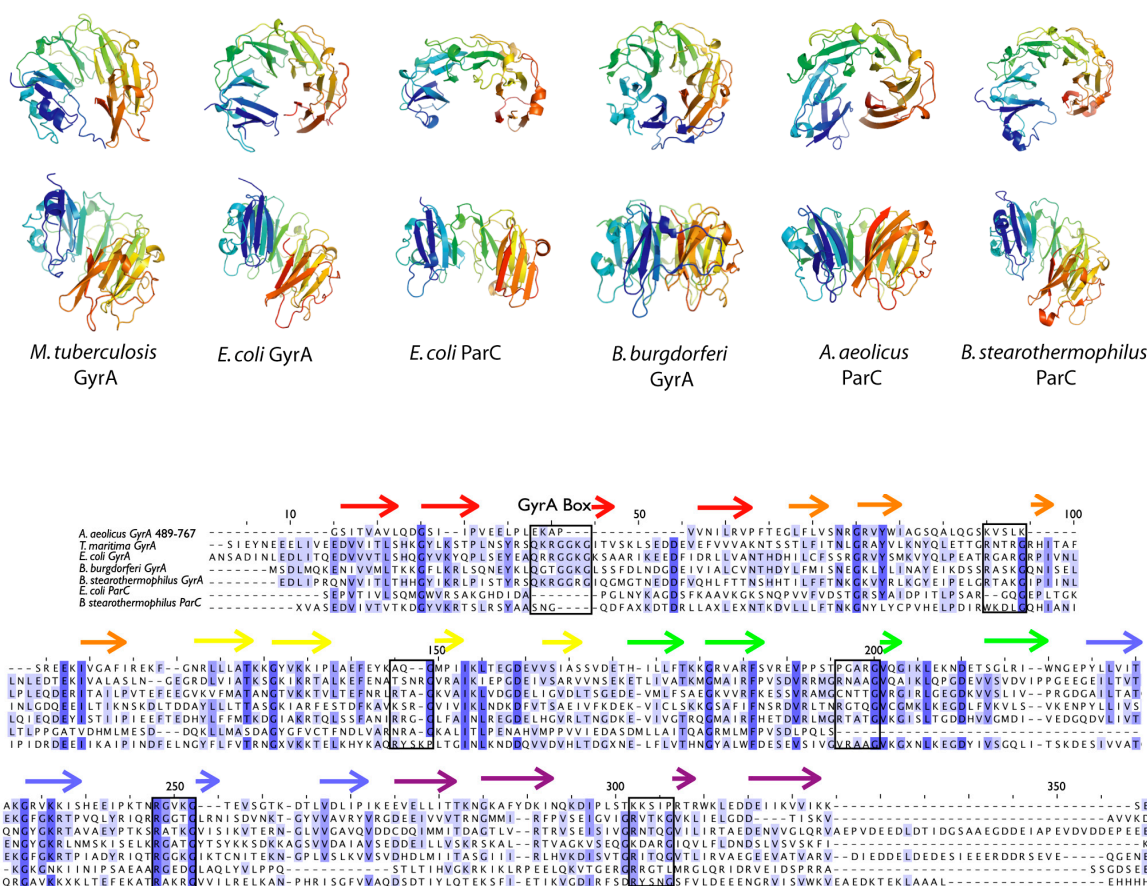


Figure 1.3. Ribbon models and sequence alignments of representative CTDs. A) Top and side views of Gyrase (GyrA) and Topo IV (ParC) C-terminal domains. Proteins are colored by rainbow (blue to red) from N- to C-terminus. B) Sequence alignment between representative CTDs. Secondary structure elements are shown with arrows above and are colored by blade. The GyrA Box in the first blade and the degenerate GyrA Box motifs in subsequent blades are boxed in black.

Chapter 2 – Crystallization of the *M. tuberculosis* Gyrase N-terminal domain

(Portions reproduced from: Tretter, E.M., Schoeffler, A.J., Weisfield, S.R., and Berger, J.M. (2009). *Proteins* 78, 492-495)

Introduction

In all cells, the topological problems associated with DNA replication, transcription and repair are managed by a class of enzymes known as DNA topoisomerases (Schoeffler and Berger 2008). Many bacteria retain a particularly unique topoisomerase, DNA gyrase, which maintains genomes in a negatively supercoiled state using an ATP-dependent duplex strand passage mechanism (Mizuuchi, Fisher et al. 1980). A key intermediate in gyrase's catalytic cycle is the formation of an enzyme-mediated, double-stranded break in which a tyrosine resident in each of the two GyrA subunits of a heterotetrameric enzyme becomes covalently attached to the DNA (Tse, Kirkegaard et al. 1980). This "cleavage complex" is potentially cytotoxic, and is stabilized by the action of clinically used antibiotics such as the fluoroquinolones (Hooper 1999).

Despite their clinical success, fluoroquinolone efficacy is being eroded by increasing levels of antibiotic resistance. In 2007, there were an estimated 9.2 million new *Mycobacterium tuberculosis* infections, 4.9% of which were caused by multidrug-resistant strains (Bottger and Springer 2008). The challenge of designing new antibiotics to combat rising resistance can be assisted by high-resolution structures of antibiotic targets; to this end, we have solved the crystal structure of the 59kDa *M. tuberculosis* DNA gyrase GyrA N-terminal domain (*MtGyrA59*), the principal drug-binding region of the enzyme. A comparative structural analysis of the *M. tuberculosis* and *E. coli* GyrA N-terminal domains reveals a previously unseen structural flexibility in a key hotspot for fluoroquinolone resistance.

Results and Discussion

At 1.6 Å, the *MtGyrA59* structure determined here is the highest resolution yet obtained for a type II topoisomerase N-terminal domain. *MtGyrA59* assembles into a heart-shaped dimer (**Figure 2.1A**), with an overall fold that closely resembles the structure of the homologous *E. coli* GyrA NTD (44% sequence identity, 1.27 Å rmsd). One notable difference between the two proteins is in the loop connecting the helix $\alpha 4$ and helix $\alpha 3$ (Gly88-Ala92), a region prone to acquiring quinolone resistance mutations. The position of this loop in *MtGyrA59* deviates significantly from its location in *E. coli* GyrA NTD and in the quinolone- and DNA- bound structure of the homologous region of *S. pneumoniae* topoisomerase IV due to crystal packing at the dimer interface (**Figure 2.1C**). Superposition of *MtGyrA59* on the topo IV fluoroquinolone-DNA structure further reveals that the position of Asp89 in *MtGyrA59* would cause a clash with moxifloxacin as bound by *S. pneumoniae* topo IV. The novel loop conformation is present in both subunits in the asymmetric unit, suggesting that this region is more conformationally dynamic than previously suspected.

MtGyrA59 and *E. coli* GyrA NTD also differ significantly in the loop encompassing Ile181 (Ile174 in *E. coli*) (**Figure 2.1D**), a region that has been shown to bend DNA in *S. cerevisiae* topoisomerase II (Dong and Berger 2007). Similar conformational diversity has been observed in other regions of the winged-helix-domain that contains this element. For example, the loop containing the active site tyrosine differs significantly between *E. coli* GyrA (residues 129-131) and the paralogous subunit of topoisomerase IV (Morais Cabral, Jackson et al. 1997; Corbett, Schoeffler et al. 2005). These examples demonstrate that the critical DNA-engaging regions of the NTD are structurally malleable, a property that may ultimately play an important role in modulating inhibitor potency. Studies of *MtGyrA* and other type II topoisomerase homologs bound to drug and DNA substrates will be needed to address this issue in the future.

Materials and Methods

Protein Purification

Residues 34-500 of *M. tuberculosis* GyrA (corresponding to residues 27-525 of the homologous *E. coli* subunit) was amplified from genomic DNA (American Type Culture Collection) and cloned into a derivative of pET28b with an N-terminal, tobacco etch virus protease-cleavable hexahistidine tag. Protein was overexpressed in *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene) by inducing log-phase cells with 0.25mM isopropyl- β -D-thiogalactopyranoside for 14 h at 25°C. Cells were harvested by centrifugation, resuspended in 20 mM Hepes pH 7.5, 800mM NaCl, 30mM imidazole, 10% Glycerol with protease inhibitors, and frozen dropwise into liquid nitrogen.

For purification, cells were sonicated and centrifuged, and the clarified lysate passed over a Ni²⁺ affinity column (Amersham Biosciences). His-tagged protein was eluted with 20mM Hepes pH 7.5, 400mM NaCl, 500mM imidazole and 10% Glycerol, and concentrated, exchanged into the same buffer containing 30mM imidazole, and incubated overnight at 4° C with hexahistidine-tagged tobacco etch virus protease (Kapust, Tozser et al. 2001). This mixture was passed over a Ni²⁺ affinity column and the flow-through purified over an S-200 gel filtration column (Amersham Biosciences) in buffer SE (50mM Hepes pH 7.5, 500mM KCl, 10% Glycerol, 2mM 2-Mercaptoethanol). Peak fractions were pooled and concentrated by ultrafiltration (Millipore Centriprep-10).

Crystallization

Purified *MtGyrA59* at 11 mg/ml was dialyzed overnight at 4° C against 10mM Hepes pH 7.5, 50mM KCl, and 1mM Tris(2-carboxyethyl)-phosphine. Crystals were grown in hanging drop format by mixing 1 μ l of protein with 1 μ l well of solution containing 26% 2-methyl-2,4-pentanediol, 100mM Tris pH 9.0, and 1mM Ciprofloxacin. Crystal reproducibility was extremely erratic, and crystal formation proved not to be drug dependent. For harvesting, crystals were transferred to a cryoprotectant solution containing well solution plus 25% glycerol for 1 minute, followed by looping and flash-frozen in liquid nitrogen.

X-ray diffraction data collection and structure determination

A single native dataset was collected on Beamline 8.3.1 at the Advanced Light Source at Lawrence Berkeley National Laboratory (Berkeley, CA). Data were indexed and reduced as P1 using HKL2000 (Otwinowski Z 1997). Phasing by molecular replacement with a poly-serine model of the *E. coli* GyrA NTD (residues 2-523, PDB 1AB4) with PHASER (Morais Cabral, Jackson et al. 1997; McCoy, Grosse-Kunstleve et al. 2007) revealed two *MtGyrA*59 protomers per asymmetric unit. Density modification and initial building were performed by PHENIX;(Adams, Grosse-Kunstleve et al. 2002) subsequent cycles of manual rebuilding and refinement were performed using COOT (Emsley and Cowtan 2004). TLS parameters were analyzed with the TLSMD server and six TLS groups were introduced in the last stages of refinement (Painter J 2006). The final model consists of residues 2-467(chain A) and 4-467(chain B) of *MtGyrA*. The final model (**Figure 2.1B**) was refined to 1.60Å resolution with a final R-factor 17.8% and R-free 20.1%. Molprobity (Lovell, Davis et al. 2003) analysis showed a total of 98.4% of residues in the most favored regions of Ramachandran space, with none in disallowed regions. (**Table 1**). The atomic structure and coordinate files have been deposited with the PDB, ID code 3ILW.

Tables

Table 2.1. Data collection and refinement statistics

Data Collection	
Beamline	ALS 8.3.1
Wavelength (Å)	1.1
Space Group	P1
Cell Dimension (Å) a, b, c	61.23, 70.05, 97.81
α, β, γ (°)	109.96, 91.16, 114.16
Resolution (Å)	50.00-1.60
R _{merge} (%) ^a	5.7 (47.8) ^b
I/σ (I)	20.6 (1.95)
Redundancy	3.6 (2.8)
Completeness (%)	99.0 (43.5)
Unique Reflections ^c	152754
Refinement	
R _{work} (%) ^d	17.8
R _{free} (%) ^e	20.1
No. atoms	
Protein	7265
Water	406
Glycerol Molecules	7
B factor (Å²)	
Protein	28.18
Water	34.89
RMSD	
Bond lengths (Å)	0.017
Bond Angles (°)	1.562
Ramachandran (%)^f	
Favored regions	98.4
Allowed regions	1.6
Outliers	0

^aR_{merge} = $\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an observation and $\langle I(hkl) \rangle$ is the mean value for its unique reflection. Summations cover all reflections.

^bThe values in parentheses indicate the highest resolution shell.

^cN_{obs}/N_{unique}.

^dR_{work} = $\sum_{hkl} ||F_{obs}| - k |F_{calc}|| / \sum_{hkl} |F_{obs}|$

^eR_{free} was calculated same way as R_{work}, but with the reflections excluded from refinement. The R_{free} set was chosen using default parameters in PHENIX (Adams, Grosse-Kunstleve et al. 2002).

^fCategories were defined by Molprobit.

Figures

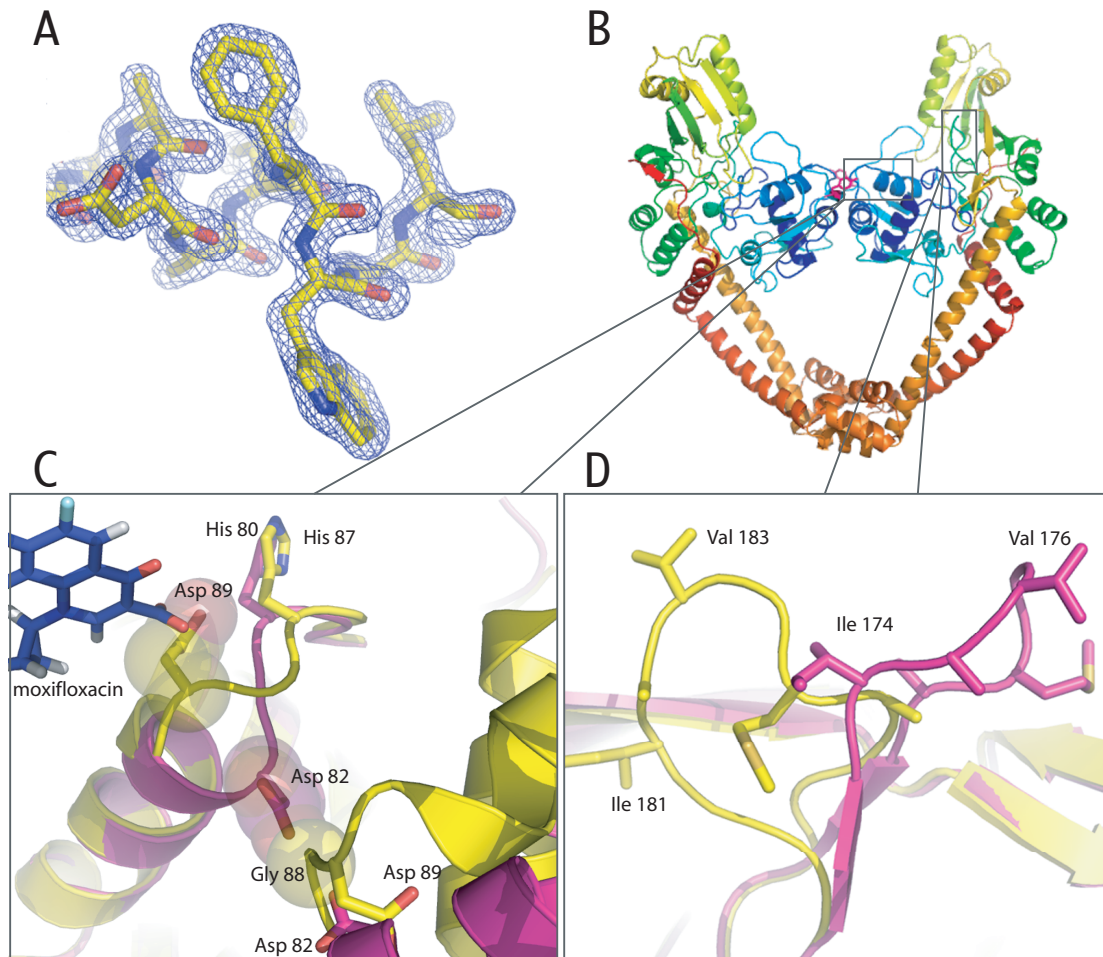


Figure 2.1 *MtGyr59* structure.

(A) Representative electron density from a refined $2F_o - F_c$ map contoured at 2σ . (B) Cartoon representation (Pymol) of the *MtGyr59* dimer. The active site Tyrosine (Tyr129) is highlighted pink. (C) Structural alignments of the *MtGyr59* (yellow) and *EcGyrA59* (fuchsia) helix $\alpha 4$ region. Gly88 and Asp82 are shown as space filling spheres to highlight the clashes that would occur if *MtGyr59* region adopted the same structure as seen in *EcGyrA59*. Asp89 is also shown, together with moxifloxacin superposed from the *S. pneumoniae* topo IV co-crystal structure, to highlight how the *MtGyr59* loop would clash with bound drug. (D) Overlay of the DNA bending loop region between *MtGyr59* (yellow) and *EcGyrA59* (fuchsia).

Chapter 3 – Mechanisms for defining the supercoiling setpoint of DNA gyrase orthologs. Part I. A non-conserved acidic C-terminal tail modulates *E. coli* gyrase activity

(Portions reproduced from: Tretter, E.M. and Berger, J.M. (2012). J Biol Chem In Press)

Introduction

DNA topoisomerases manage chromosome supercoiling and organization in all cells. Gyrase, a prokaryotic type IIA topoisomerase, consumes ATP to introduce negative supercoils through a strand passage mechanism. All type IIA topoisomerases employ a similar set of catalytic domains for function; however, the activity and specificity of gyrase is augmented in by a specialized DNA binding and wrapping element, termed the C-terminal domain (CTD), which is appended to its GyrA subunit (**Figure 3.1A**). We have discovered that an unconserved, acidic tail at the extreme C-terminus of the *Escherichia coli* (*E. coli*) GyrA CTD has a dramatic and unexpected impact on gyrase function. Removal of the CTD tail enables GyrA to introduce writhe into DNA in the absence of GyrB, an activity exhibited by other GyrA orthologs, but not by wild-type *E. coli* GyrA. Strikingly, a “tail-less” gyrase holoenzyme is markedly impaired for DNA supercoiling capacity, but displays normal ATPase function. Our findings reveal that the *E. coli* GyrA tail regulates DNA wrapping by the CTD to increase the coupling efficiency between ATP turnover and supercoiling, demonstrating that CTD functions can be fine-tuned to control gyrase activity in a highly sophisticated manner.

In comparing the supercoiling properties of *Mycobacterium tuberculosis* (*Mtb*) and *E. coli* gyrase, we discovered that the isolated GyrA proteins of the two species differ dramatically in their respective abilities to wrap DNA (see Chapter 4). Further analysis of this distinction led us to probe the function of the unconserved stretch of amino acids that follows the CTD in *E. coli* GyrA (**Figure 3.1B**). We unexpectedly found that removal of either the entirety or an internal portion of the tail bestows *E. coli* GyrA with the capacity to wrap DNA, an activity exhibited by MtbGyrA, but not by the wild-type *E. coli* protein. The isolated, full-length *E. coli* GyrA CTD also is unable to wrap, or even bind DNA, whereas ablating or trimming the tail restores these functions. Interestingly, alterations to the CTD tail have no effect on either basal or DNA-stimulated ATPase activity, but greatly reduce both the rate of negative supercoiling and the final level of superhelical density that can be introduced by gyrase. These findings indicate that species-specific appendages to the GyrA CTD can regulate its function in the context of the gyrase holoenzyme, thereby ensuring that ATP turnover is tightly coupled to supercoiling efficiency.

Results and Discussion

Control of DNA wrapping by the negatively-charged *E. coli* GyrA tail

The ability of gyrase to wrap large segments of DNA (~120-140bp) has been well established for over thirty years (Liu and Wang 1978). Wrapping is known to be a property of the GyrA subunit and its C-terminal domain (Reece and Maxwell 1991; Ruthenburg, Graybosch et al. 2005); however, biochemical studies of *E. coli* GyrA by Maxwell and colleagues have shown that the full-length protein is unable to introduce writhe into DNA on its own (Reece and Maxwell 1991). Although this finding at the time did not elicit additional investigation, it is surprising in retrospect, as several studies have shown that isolated GyrA CTDs are readily capable of wrapping DNA, in some instances by nearly a full turn (Reece and Maxwell 1991; Corbett, Shultzaberger et al. 2004; Ruthenburg, Graybosch et al. 2005; Hsieh, Yen et al. 2010).

The dichotomy between the wrapping behaviors of *E. coli* GyrA and its CTD came to our attention when we found that Mtb GyrA imparts writhe on its own as efficiently as its isolated CTD (see Chapter 4). In the course of investigating this distinction, we realized that one source of variation between the two species' CTDs, and between bacterial GyrA proteins in general, resides in the extreme C-terminus of the subunit (**Figure 3.1B**). This element, referred to hereafter as the GyrA "tail" is highly acidic in both *E. coli* and Mtb GyrA (the predicted pI of each is ~3.0), but is significantly shorter in the latter. In other species, we found that the GyrA tail greatly varies in length, or is absent entirely (e.g. *B. burgdorferi* and *T. maritima*). We might have dismissed these small differences as trivial, were it not for a second source of variation that came to our attention: namely, every published study concerning the wrapping behavior of the isolated *E. coli* GyrA CTD has been conducted with constructs that lack a portion of the full-length domain, several of which include tail truncations (Reece and Maxwell 1991; Ruthenburg, Graybosch et al. 2005; Hsieh, Yen et al. 2010).

To determine if the tail plays a role in modulating wrapping by *E. coli* gyrase, we first examined its effect on the biochemical properties of the isolated CTD and of GyrA. Secondary structure predictions using PSIPRED indicate that the GyrA CTD tails are disordered (not shown), explaining why they have been either truncated or unobservable in the available crystal structures (McGuffin, Bryson et al. 2000; Ruthenburg, Graybosch et al. 2005; Hsieh, Yen et al. 2010) (see also Chapter 4). We therefore created a series of tail deletions that lacked either an internal acidic segment, or the entirety of the unconserved region.

We initially assessed the ability of the CTD to wrap DNA using topology footprinting. In this assay, the amount of writhe imparted by a protein is assessed by incubating varying amounts of the protein with nicked plasmid DNA; the subsequent addition of ligase seals the nicked plasmid, after which treatment with SDS/proteinase K removes bound molecules to leave the DNA with an "afterimage" of the number of supercoils originally constrained upon binding. Notably, the full-length *E. coli* GyrA CTD was completely unable to introduce any writhe into plasmid DNA (**Figure 3.2A**). By contrast, both the insert-less CTD and complete tail deletion exhibited the robust DNA wrapping activity seen by other groups. A similar examination of *E. coli* GyrA reproduced the lack of observable wrapping by the full-length protein as first noted by

Maxwell and coworkers (**Figure 3.2B**). As with the CTD alone, removal of the internal tail region resulted in extensive wrapping by the GyrA truncated construct (**Figure 3.2B**), as did complete ablation of the tail (not shown).

Regulation of DNA binding by the GyrA tail

Because removal of the GyrA tail actually led to a *gain* of wrapping function, we surmised that our alterations were not impairing the activity of the CTD, but rather eliminating an internal repressive element. The fact that the CTD responded to the deletions in a manner analogous to full-length GyrA in turn implied that at least part of the control was at the level of the CTD itself, and not simply due to an ability of GyrA to sequester the CTD from DNA in a tail-dependent manner.

To test this idea further, we set out to compare the abilities of the wild-type and truncated CTDs to bind DNA using fluorescence anisotropy. In this assay, short, fluorescently labeled, DNA oligos were incubated with varying amounts of each purified *E. coli* CTD construct, and binding affinity was measured as a function of the change in the relative amount of circularly polarized light that is emitted by the sample (Lakowicz 2006). Analysis of the data from this assay showed clearly that both the tailless and insert-less CTD constructs were competent to bind the DNA oligo with similar affinities ($K_{d, app} = 90 \text{ nM} \pm 21 \text{ nM}$ and $42 \text{ nM} \pm 8 \text{ nM}$, respectively) (**Figure 3.3**). By contrast, no binding was detectable for the full-length CTD. These results demonstrate that the tail directly blocks the ability of the GyrA CTD to engage DNA, and that its removal relieves this autoinhibitory function.

Effects of tail on DNA wrapping by the gyrase holoenzyme

Since the *E. coli* gyrase holoenzyme readily wraps DNA (Rau, Gellert et al. 1987), and because GyrA alone is unable to do so (Reece and Maxwell 1991), we reasoned that the binding of GyrB might be capable of relieving the repressive action of the CTD tail. An additional line of evidence supporting this idea comes from a study showing that wild-type *E. coli* gyrase is unable to introduce writhe into DNA in the presence of AMP-PNP (Kampranis, Bates et al. 1999; Heddle, Mitelheiser et al. 2004). As AMP-PNP is known to act through the GyrB subunits, triggering their dimerization (Wigley, Davies et al. 1991), this result suggests that a nucleotide-actuated switch in the ATPase subunits might control wrapping; however, the molecular mechanism of this switch has not been established.

To test whether the GyrA tail might be a control linkage affected by the nucleotide status of GyrB, we measured the ability of tail-truncated *E. coli* gyrase holoenzyme to introduce writhe in the presence and absence of AMP-PNP. Because both the insert-less and tailless CTD constructs display similar biochemical properties, we investigated this phenomenon with the more conservative, insert-less GyrA construct. To capture a “topology footprint” by gyrase, an ATP-independent assay technique must be utilized to prevent the enzyme from supercoiling the plasmid DNA substrate. We therefore incubated gyrase with relaxed plasmid DNA and eukaryotic topoisomerase IB, which relaxes supercoils in the absence of ATP (Champoux and Dulbecco 1972; Shuman 1998). As seen previously (Heddle, Mitelheiser et al. 2004), wild-type gyrase strongly wraps DNA in the absence of nucleotide, and loses the ability to wrap when AMP-PNP is present (**Figure 3.4**). By contrast, removal of a portion of the tail allowed gyrase to

wrap DNA regardless of whether nucleotide was present. Interestingly, the degree of wrapping exhibited by the insert-less holoenzyme was equivalent under AMP-PNP-free and -bound conditions, but significantly less than that manifested by apo wild-type gyrase. These findings demonstrate that the acidic GyrA tail is necessary for GyrB to regulate DNA wrapping by the *E. coli* holoenzyme, and that the tail allows for a more significant introduction of writhe when ATP is absent.

Impact of the GyrA tail on DNA supercoiling by gyrase

Given the marked effect on the control of DNA wrapping by the GyrA tail, we next assayed the impact of tail deletions on strand passage. To assess this function, we reconstituted tetrameric gyrase using either insert-less or wild type GyrA, together with wild-type GyrB, and compared titrations of the mutant and wild-type enzymes in gyrase activity assays. We first looked at the ATP-dependent introduction of negative supercoils into relaxed plasmid DNA using native agarose gel electrophoresis over a range of holoenzyme concentrations. Notably, comparison of wild-type and insert-less gyrase showed that deletion of the CTD tail insert reduced the specific activity of supercoiling by more than 50-fold (**Figure 3.5A**, upper). Deletion of the entire CTD tail produced a similar effect to that seen by the insert-less mutant (not shown). Since the insert-less GyrA protein used in this assay binds and wraps DNA extensively on its own (**Figure 3.2B**), and since the same GyrB preparation was used for both the mutant and wild-type supercoiling experiments, the most parsimonious explanation for this effect is that the GyrA tail is necessary for the robust activity normally displayed by the full-length *E. coli* holoenzyme.

To gain more insights into this reduction in catalytic ability, we asked whether the supercoiled DNA produced by the wild-type and insert-less enzymes was underwound to a similar extent. This property was assessed by running the same supercoiling reactions on agarose gels in the presence of 3 $\mu\text{g/ml}$ chloroquine. Chloroquine is a weak intercalating agent that overwinds DNA to permit the separation of supercoiled topoisomers; at appropriate concentrations, chloroquine can help resolve negatively-supercoiled species that would otherwise cluster in a single high-mobility band on a native gel (Clark and Leblanc 2009). Analysis of the topoisomer distribution by this method shows that the activity of the insert-less enzyme gave rise to DNA species that were much less negatively-supercoiled than the products of wild-type gyrase (**Figure 3.5A**, lower).

To discern whether the apparent reduction in supercoiling levels attained by the insert-less gyrase was due to a slow rate of enzyme turnover, which could prohibit the reaction from going to completion within our standard reaction time (30 min), we assayed supercoiling over a range of different periods. The reconstituted mutant *E. coli* gyrase was added to relaxed plasmid DNA and ATP at a concentration where complete supercoiling was seen in our enzyme titration experiments (20 nM), and incubated at 37° C for 5, 10, 30, and 90 min; wild-type *E. coli* gyrase was compared as a control. Time points that exceeded 30 minutes were supplemented with additional ATP to prevent ATP-independent relaxation events from occurring, and the results of this assay were again visualized using agarose gel electrophoresis in the absence and presence of 3 $\mu\text{g/ml}$ chloroquine. We found that even when given extremely long amounts of time, the insert-less *E. coli* gyrase was unable to negatively supercoil the plasmid DNA to the same

extent as the wild-type enzyme (**Figure 3.5B**). Interestingly, the degree of supercoiling manifested by the mutant approximated that seen for wild-type gyrase at the shortest time assayed (5 min), indicating that deletion of the tail did not simply slow the rate of strand passage. Rather, this element appears to have a direct and profound effect on the superhelical density “setpoint” of the enzyme.

In addition to negatively supercoiling DNA, gyrase can relax negatively-supercoiled DNA in an ATP-independent manner (Gellert, Mizuuchi et al. 1977). Although the physiological role of this reaction is unclear, it does provide another window into understanding the overall mechanism of the enzyme. Moreover, other type II topoisomerases such as topo II and topo IV fail to exhibit ATP-independent relaxation, suggesting that the unique DNA wrapping properties of gyrase may play a role in this activity. To assess the effect of the tail on the relaxation of negatively-supercoiled DNA by gyrase, we titrated different amounts of either the insert-less mutant or wild-type gyrase against a fixed amount of DNA in the absence of ATP. After 30 min, the products of this reaction were visualized using native agarose gel electrophoresis. As with supercoiling, the insert-less mutant proved to be significantly less active (>10-fold) than wild-type enzyme. This finding indicates that either robust wrapping by the gyrase holoenzyme, the control of wrapping, or both is critical to its ATP-independent relaxation activity (**Figure 3.6**).

Effect of the tail on gyrase ATPase activity

Because GyrB and its ATPase status were the only factors previously known to variably modulate DNA wrapping by *E. coli* gyrase, we next asked if the tail was an important factor in nucleotide turnover. To address this question, the ATPase activities of wild-type and insert-less *E. coli* gyrase were assessed using a malachite green colorimetric assay in which ATP hydrolysis is measured by phosphate release (Lanzetta, Alvarez et al. 1979). We first conducted these measurements by incubating reconstituted gyrase with increasing amounts of ATP at a fixed concentration of sheared salmon-sperm DNA; the addition of DNA is known to dramatically stimulate the ATPase activity of the enzyme (**Figure 3.7A**) (Maxwell and Gellert 1984). We then varied the amount of DNA present in the reaction while holding the initial concentration of ATP constant (**Figure 3.7B**). Finally, we performed the reactions for different amounts of time using a fixed starting concentration of DNA and ATP. In all instances, the insert-less gyrase hydrolyzed ATP comparatively to wild-type gyrase, demonstrating that alteration of the GyrA tail does not have an adverse effect on ATP turnover by gyrase. The findings further argue against the observed loss of supercoiling activity by the insert-less mutant as having arisen from simple inactivation of the enzyme. Instead, given our observation that gyrases lacking some or all of the tail are impaired for DNA wrapping and supercoil introduction in the context of the holoenzyme (but not with the isolated GyrA subunit or CTD), these data suggest that the tail is a linkage element that allows gyrase to efficiently couple ATP turnover to productive strand passage events.

Conclusion

In this chapter, we have uncovered a new control element that both promotes turnover efficiency and helps lock the “setpoint” for DNA supercoiling by an archetypal DNA remodeling enzyme, *E. coli* gyrase. Like any molecular machine, gyrase is

composed of multiple moving parts and complementary activities that collaborate to support function. We have found that a non-conserved, acidic tail appended to GyrA regulates DNA binding and wrapping by an auxiliary domain at the C-terminus of the subunit. The deletion of small internal segments of the tail, or its ablation, actually permit the CTD to engage and deform DNA, either on its own or in the context of GyrA, indicating that the tail is an auto-inhibitory element (**Figures 3.2 & 3.3**). It has been shown previously that GyrB can both restore or re-inhibit DNA wrapping by GyrA, depending on its ATPase status (Hedde, Mittelheiser et al. 2004); we now find that this regulation depends on the presence of a fully intact GyrA tail (**Figure 3.4**). Strikingly, removal of the tail decreases the rate and extent of DNA underwinding by gyrase, but does not significantly affect its ATP hydrolysis properties (**Figures 3.5 & 3.7**). Hence, the tail helps to ensure that the consumption of ATP is tightly coupled to rapid and efficient strand passage.

How might the tail exert these effects physically? The finding that the negatively-charged tail prevents the isolated CTD from binding DNA suggests that it acts in *cis*, perhaps by associating electrostatically with the rim of the domain, which is positively charged in all such domains imaged to date (Corbett, Shultzaberger et al. 2004; Ruthenburg, Graybosch et al. 2005; Hsieh, Yen et al. 2010). This type of interaction could block DNA from accessing the CTD through steric occlusion; removal of small segments of the tail might prevent this interaction from forming stably. Alternatively, or in addition, the tail might help sequester the CTD away from DNA, perhaps by linking the domain to the rest of the GyrA subunit. The binding of GyrB could potentially relieve auto-inhibition of the CTD directly, by engaging the tail to allow DNA wrapping, or allosterically, by inducing a conformational change in GyrA that promotes both CTD release and undocking of the tail to permit DNA engagement.

A scheme outlining these concepts is shown in **Figure 3.8**. Importantly, it makes several predictions that are supported by various lines of experimental evidence. For instance, small X-ray scattering studies have suggested previously that the *E. coli* CTD may associate with the rest of GyrA in a manner that would prevent it from readily engaging a DNA bound to the active site region of the protein (Costenaro, Grossmann et al. 2005; Baker, Weigand et al. 2011). Our deletion studies demonstrate that the tail is not only critical for the GyrB-dependent modulation of DNA wrapping by gyrase, but that the extent of holoenzyme-induced wrapping is compromised when the tail is altered (**Fig. 4**). Given that partial or complete truncation of the tail enables robust wrapping by both the CTD and GyrA on their own (**Figure 3.2**), these data suggest that the presence of GyrB helps place the CTD in a productive conformation to bind DNA, introduce writhe, and correctly position a DNA segment in *cis* for strand passage. Direct tethering of the tail to GyrB would link wrapping to the ATPase cycle, and help ensure that turnover and strand passage are efficiently interconnected. Both coupling phenomena have been seen biochemically (Sugino and Cozzarelli 1980; Cullis, Maxwell et al. 1992; Roca and Wang 1992; Kampranis, Bates et al. 1999), although the CTD tail has not been implicated in the process until now.

Materials and Methods

Protein purification

A truncated *E. coli* GyrA CTD construct (residues 531-853) (Reece and Maxwell 1991; Ruthenburg, Graybosch et al. 2005), as well as full-length *E. coli gyrA* (1-875) and *gyrB* (1-804) genes were cloned into pET28b. The full-length *E. coli* GyrA CTD (531-875), along with “insert-less” *E. coli gyrA* and the GyrA CTD (both missing residues 842-856), were amplified from the *gyrA* pET28b vector and cloned into a derivative of pET28b using an in-house ligation independent cloning vector system (pLIC) behind an N-terminal, tobacco etch virus (TEV) protease-cleavable hexahistidine tag. Proteins were expressed in *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene) by inducing log-phase cells grown in 2xYT broth with 0.25 mM isopropyl- β -D-thiogalactopyranoside overnight at 18°C. Cells were harvested by centrifugation, resuspended in 20 mM Tris-HCl pH 7.9, 800 mM NaCl, 30 mM imidazole, 10% glycerol, and protease inhibitors (1 μ M leupeptin, 1 μ M pepstatin A, and 1 mM phenylmethylsulfonyl fluoride), and then frozen dropwise in liquid nitrogen for storage at -80°C.

For purification, cells were sonicated and centrifuged, and the clarified lysate was passed over a Ni²⁺-affinity column (Amersham Biosciences). The his-tagged protein was eluted with 20mM Tris-HCl pH 7.9, 100 mM NaCl, 500 mM imidazole, 10% glycerol, and protease inhibitors (1 μ M leupeptin, 1 μ M pepstatin A, and 1 mM phenylmethylsulfonyl fluoride), concentrated (Millipore Amicon Ultra- 10/30) and exchanged into the same buffer containing 30 mM imidazole, then incubated overnight at 4°C with 1-1.5 mg hexahistidine-tagged TEV protease (Kapust, Tozser et al. 2001). Following TEV cleavage, the mixture was passed over a Ni²⁺-affinity column, and the flow-through collected, concentrated, and passed over an S-200 or S-300 gel filtration column (Amersham Biosciences) in 50 mM Tris-HCl pH 7.9, 500 mM KCl, 10% glycerol, and 2 mM 2-mercaptoethanol. Peak fractions (determined by UV absorbance) were pooled and concentrated by centrifugal filtration (**Fig. S3.1**) (Millipore Amicon Ultra-10/30).

Topological footprinting assays

The introduction of DNA writhe by various gyrase domains and subunits was assessed in a buffer containing 15 mM Tris-HCl pH 7.5, 13% glycerol, 6 mM MgCl₂, 0.1 mg/ml BSA, 70 mM KCl, and 300 ng (6 nM) nicked pSG483 as a DNA substrate. Varying amounts of protein were added to reaction mixtures (30 μ L total volume) and equilibrated at 37°C for 20 minutes, after which 60 units of ligase and 1 mM ATP were added. Reactions were allowed to incubate at 37°C for an additional 30 min, and then stopped by the addition of SDS (1% final concentration), EDTA (10 mM final concentration), and proteinase K (50 μ g/ml final concentration), followed by incubation at 37°C for 30 additional minutes. Reactions were analyzed by electrophoresis through 1.0% agarose gels (Invitrogen) with 1x TAE running buffer. Gels were run at 1.7 V/cm for 19-21 hours, stained with ethidium bromide (EtBr), and visualized by UV trans-illumination.

To capture a gyrase holoenzyme “topology footprint”, we used an ATP-independent assay technique to prevent gyrase from supercoiling the plasmid DNA

substrate. In these reactions, gyrase holoenzyme was incubated with relaxed plasmid and eukaryotic topoisomerase IB, which relaxes DNA supercoils in the absence of ATP (Champoux and Dulbecco 1972; Shuman 1998). Holoenzyme write assays were performed in the presence and absence of 2 mM AMP-PNP using relaxed pSG483 plasmid and topoisomerase IB purified in house from wheat germ (Dynam, Jendrisak et al. 1981).

DNA binding assays

DNA binding was determined by fluorescence anisotropy using a random, 37-base pair segment with 40% GC content as a substrate. Annealed oligos 5'- TAA AGT CTA GAG ACA CGC ATA GTC AAT GAC GGA GTT A -3' and 5'- |56-FAM|TAA CTC CGT CAT TGA CTA TGC GTG TCT CTA GAC TTT A -3' (where 56-FAM indicates the position of a carboxyfluorescein dye for visualization) were purchased from Integrated DNA Technologies and resuspended in TE Buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0). Varying amounts of *E. coli* GyrA full-length CTD, insert-less CTD and tail-less CTD were incubated with 20 nM of fluorescently labeled duplex oligonucleotide at room temperature in the dark in 20 mM Tris-HCl pH 7.5, 70 mM KCl, 10% glycerol, and 1 mM MgCl₂. Fluorescence anisotropy measurements were performed using a Victor 3V (Perkin Elmer) multi-label plate reader. Data points represent the average of three independent measurements, where error bars represent the standard deviation between measurements. Binding curves were fit to a simplified version of the single site binding equation that holds for our experimental conditions using Kaleidagraph version 4.0 (Synergy software):

$$\theta = \frac{[L]}{K_{d,app} + [L]}$$

where θ represents the fraction of ligand binding sites filled, $K_{d,app}$ is the apparent dissociation constant, and L is the ligand concentration.

DNA relaxation and supercoiling assays

pSG483, a derivative of pUC19 containing a unique Nb.BbvCI nicking site, was used for supercoiled and relaxed DNA substrates. Negatively-supercoiled plasmid was purified from *E. coli* with a maxiprep kit (Macherey Nagel). Relaxed plasmid was made using nick-ligation by first nicking maxiprepplasmid DNA with Nb.BbvCI and then ligating with T4 DNA ligase. DNA supercoiling or relaxation assays (30 μ L) were performed in a buffer containing 15 mM Tris-HCl pH 7.5, 13% glycerol, 6 mM MgCl₂, 0.1 mg/ml BSA, 70 mM KCl, 1 mM dithioerythritol, 2 mM ATP and 300 ng (6 nM) DNA substrate; ATP was omitted to assess nucleotide-independent relaxation. Varying amounts of reconstituted holoenzyme were added to reaction mixtures, and reactions were allowed to proceed for 30 minutes at 37°C. Time course reactions contained 4 mM ATP; for times longer than 30 min, 2 mM ATP was further added after 60 min. Reactions were stopped by the addition of SDS (1% final concentration) and EDTA (10 mM final concentration), and were analyzed by electrophoresis through 1.0% agarose gels with 1x TAE running buffer. Chloroquine gels contained an additional 3 μ g/ml chloroquine (Sigma) in the gel and running buffer. Gels were run at 1.7-5.0 V/cm for 4-12 hours, stained with ethidium bromide (EtBr), and visualized by UV trans-illumination.

ATPase assays

ATPase assays were performed using a malachite green/molybdate assay for free phosphate. Reactions were run under supercoiling assay conditions and contained 50 nM gyrase. In the ATP titration assay, sheared salmon sperm DNA was held constant at 50 μ M and reactions were supplemented with a titration of ATP (0 to 4 mM). The DNA titration assays were started with 2 mM ATP and were supplemented with varying amounts of sheared salmon sperm DNA (0 to 100 μ M). ATP was added last to initiate the reactions, followed by a 30 min incubation at 37°C. 200 μ L of malachite green reagent (0.034% malachite green, 10 mM ammonium molybdate, 1 N hydrochloric acid, 3.4% ethanol, and 0.01% Tween 20) was then added, followed by a 5 min incubation at room temperature in the dark. Malachite green reagent was made fresh daily and sterile filtered before use. Absorbance at 620 nm was measured using a Perkin-Elmer Victor3V plate reader. All reactions were performed in triplicate. The ATP titration data were fit to the Michaelis-Menten model, $v = (v_{\max} [S]) / (K_m + [S])$ where v is the reaction rate, $v_{\max}$ is the maximum rate, $[S]$ is the total enzyme concentration, and K_m is the Michaelis-Menten constant. The DNA titration data were fit to the Hill equation, $v = v_{\max} [S]^h / (K_d^h + [S]^h)$ where v is the reaction rate, $v_{\max}$ is the maximum rate, $[S]$ is the total enzyme concentration, h is the Hill coefficient, and K_d is the apparent dissociation constant.

Figures

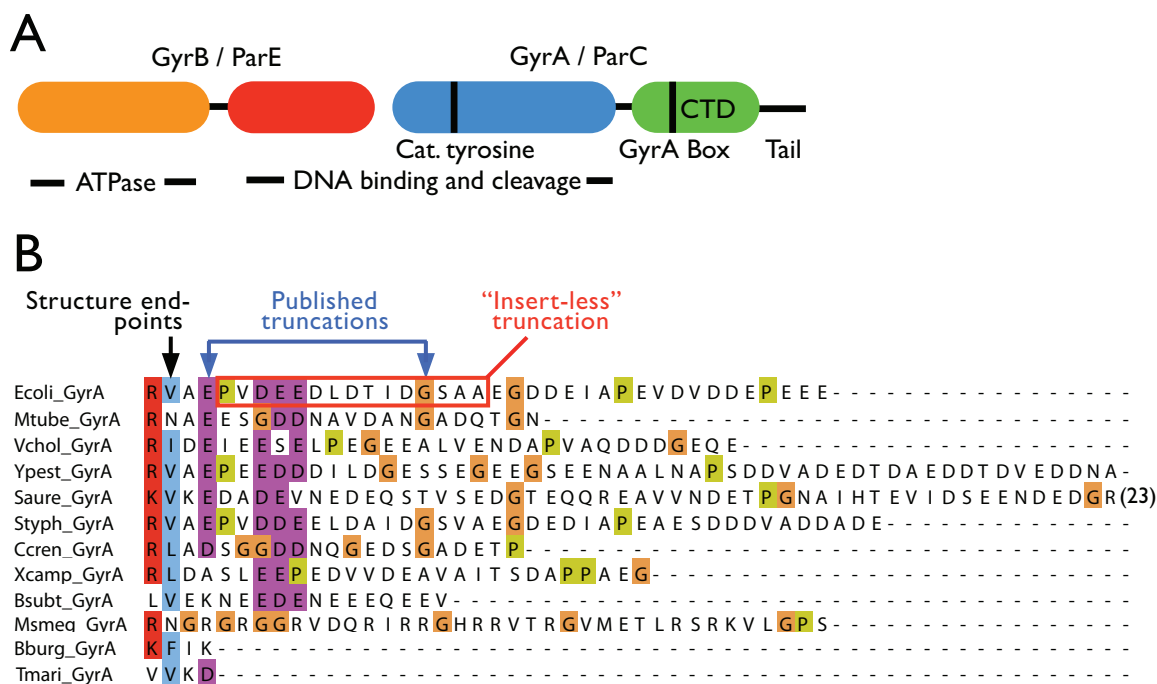


Figure 3.1. Bacterial type IIA topoisomerase structure and tail alignment.

(A) Primary domain structure of bacterial type IIA topoisomerases. Catalytic functions are indicated with horizontal bars. Vertical black bars mark the relative locations of the catalytic tyrosine and the GyrA box. (B) Sequence alignment between representative CTD tails. GyrA residue numbers are as follows: *E. coli* GyrA 838-875; *M. tuberculosis* GyrA 817-838; *V. cholera* GyrA 862-894; *Y. pestis* GyrA 838-891; *S. aureus* GyrA 809-886; *S. typhimurium* GyrA 838-878; *C. crescentus* GyrA 899-919; *X. campestris* GyrA 872-899; *B. subtilis* GyrA 805-821; *M. smegmatis* GyrA 817-854; *B. burgdorferi* GyrA 807-810; and *T. maritima* GyrA 801-804.

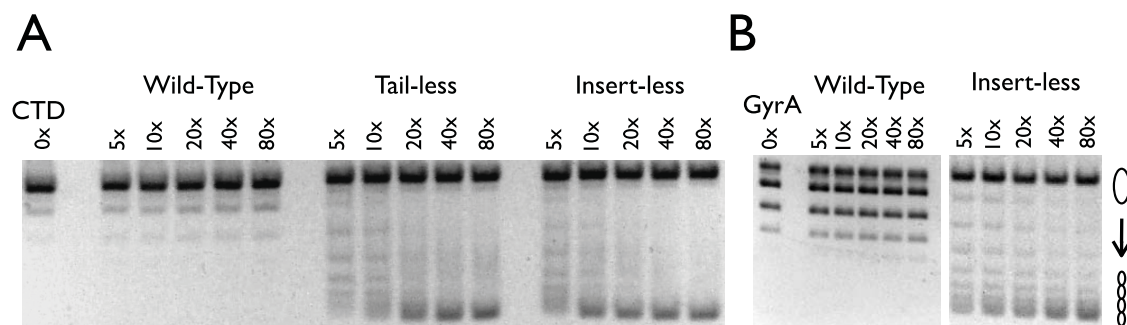


Figure 3.2. GyrA and CTD topology footprint assays. (A) Nicked pSG483 (6 nM) was incubated with varying amounts of the *E. coli* GyrA CTD (indicated as a molar excess of CTD over plasmid) and ligase. (B) Nicked pSG483 (6 nM) was incubated with varying amounts of *E. coli* GyrA dimer (indicated as a molar excess of GyrA dimer over plasmid) and ligase.

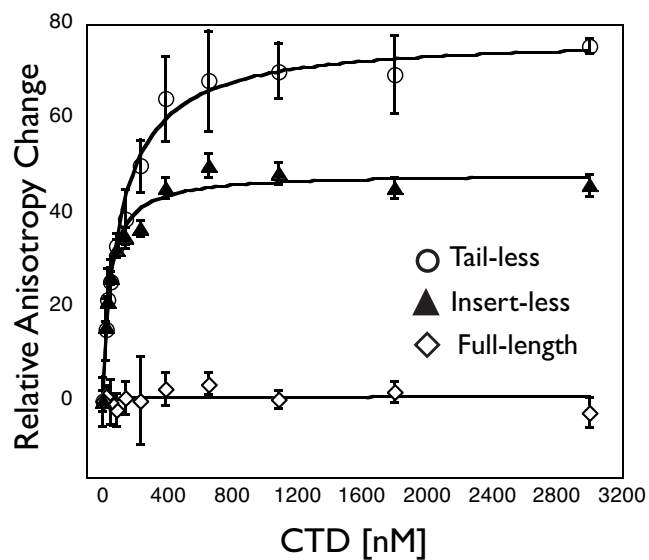


Figure 3.3. DNA binding by CTD constructs. Binding of a fluorescein-tagged, double-stranded 37-mer oligonucleotide (20 nM), monitored as a function of CTD concentration by fluorescence anisotropy. Y-axis units in mA. The apparent K_d values are as follows: Tail-less 90 nM $\pm$ 21 nM; Insert-less 42 nM $\pm$ 8 nM; Full-length n/d.

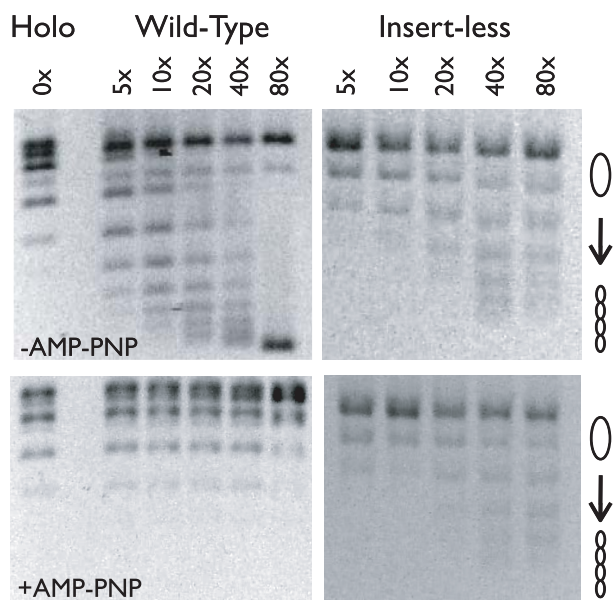


Figure 3.4. Gyrase holoenzyme topology footprint assays. Relaxed pSG483 (6 nM) was incubated with varying amounts of reconstituted holoenzyme and topo IB in the absence (top panel) and presence (bottom panel) of AMPPNP (2 mM) (indicated as a molar excess of holoenzyme over plasmid). The positions of relaxed and negatively-supercoiled DNA species are labeled with cartoon representations on the right.

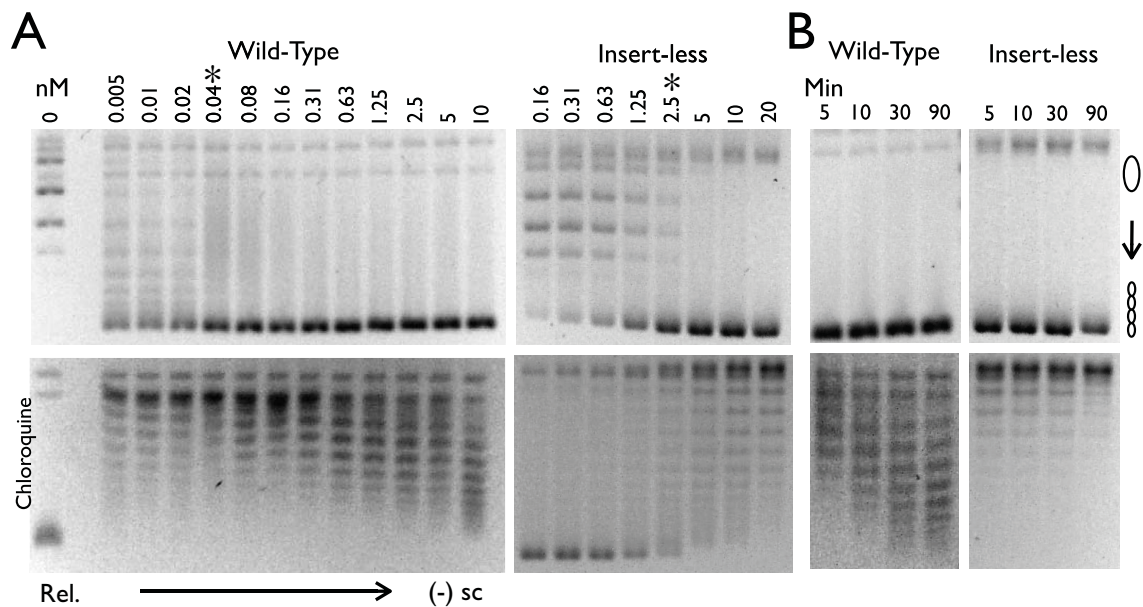


Figure 3.5. Comparison of supercoiling extent by wild-type and insert-less *E. coli* gyrase. (A) Negative supercoiling activity. Protein concentrations are listed in nM holoenzyme and asterisks denote the minimal concentrations of enzyme needed to supercoil the substrate in 30 min. (B) Negative supercoiling time course assay using 20 nM holoenzyme. Time points are listed in minutes. A portion of each sample was run on a 1% agarose gel in the absence and presence (bottom) of 3 $\mu\text{g/mL}$ chloroquine. DNA topoisomers are labeled with cartoon representations across the bottom of the chloroquine gel. Relaxed and supercoiled DNA species are labeled with cartoon representations on the right of the native gel. All reactions were started with 6 nM relaxed pSG483 plasmid DNA substrate.

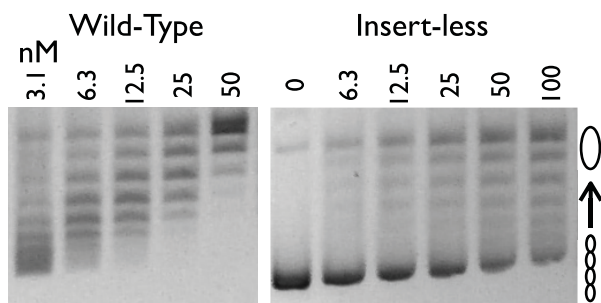


Figure 3.6. ATP-independent, negative supercoil relaxation by wild-type and insert-less *E. coli* gyrase holoenzyme. The positions of relaxed and negatively-supercoiled DNA species are labeled with cartoon representations on the right. All reactions were started with 6 nM pSG483 plasmid DNA substrate.

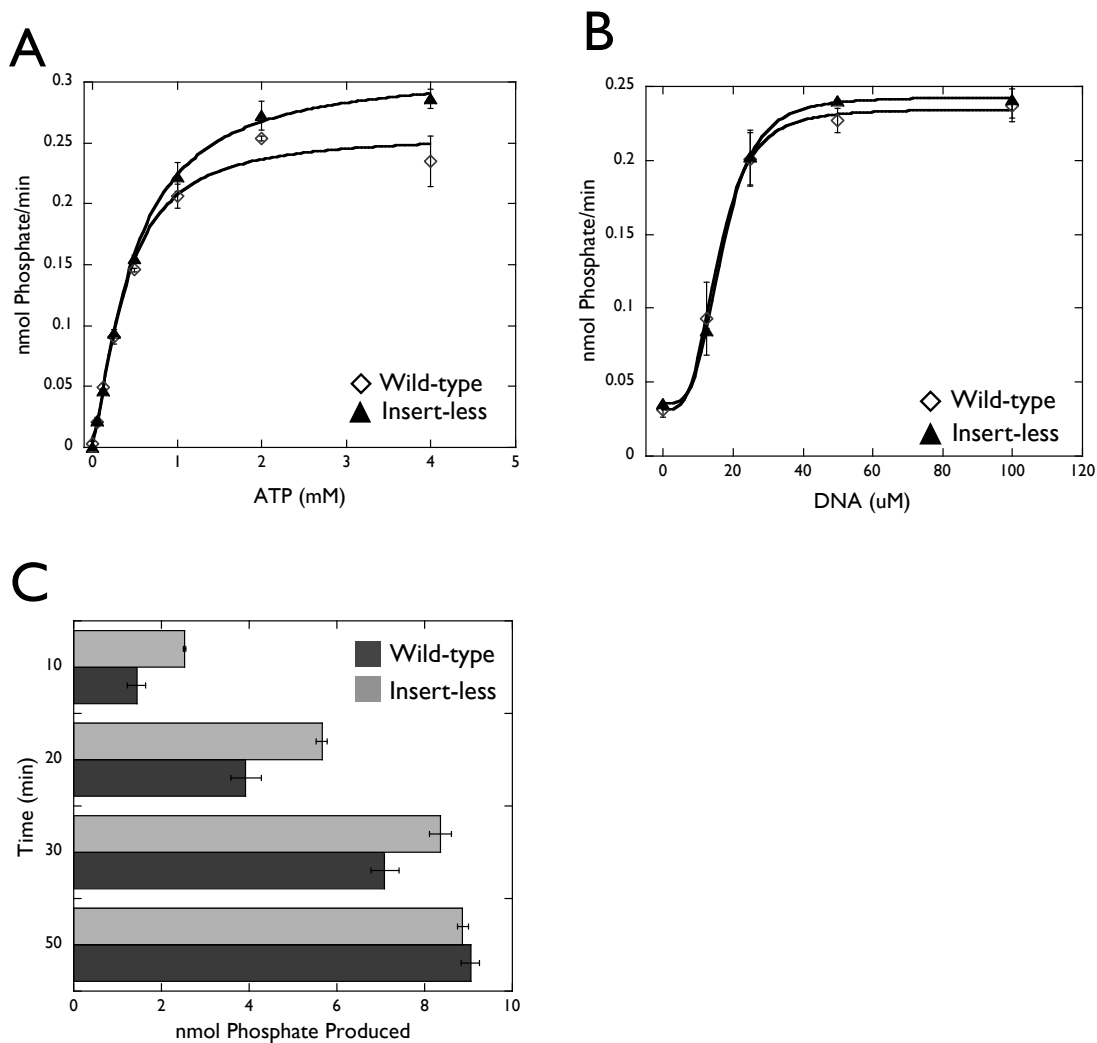


Figure 3.7. ATP hydrolysis by wild-type and insert-less *E. coli* gyrase. (A) Basal ATPase activity plotted as a function of starting ATP concentration in mM (x-axis) and nmol phosphate produced per minute (y-axis). Data were fit to an apparent Michaelis-Menten model ($R_{wt}=0.996$; $R_{\Delta tail}=1.000$). (B) DNA stimulated ATPase activity plotted as a function of sheared salmon-sperm DNA concentration in μM (x-axis) and nmol phosphate produced per minute (y-axis). Data were fit to the Hill equation ($R_{wt}=1.000$; $R_{\Delta tail}=1.000$). (C) ATPase activity time course. Wild-type and insert-less *E. coli* gyrase ATPase activities are plotted as a function of nmol phosphate produced (x-axis) and time in minutes (y-axis). Phosphate produced during 50 min time course is approaching the saturation point for the assay.

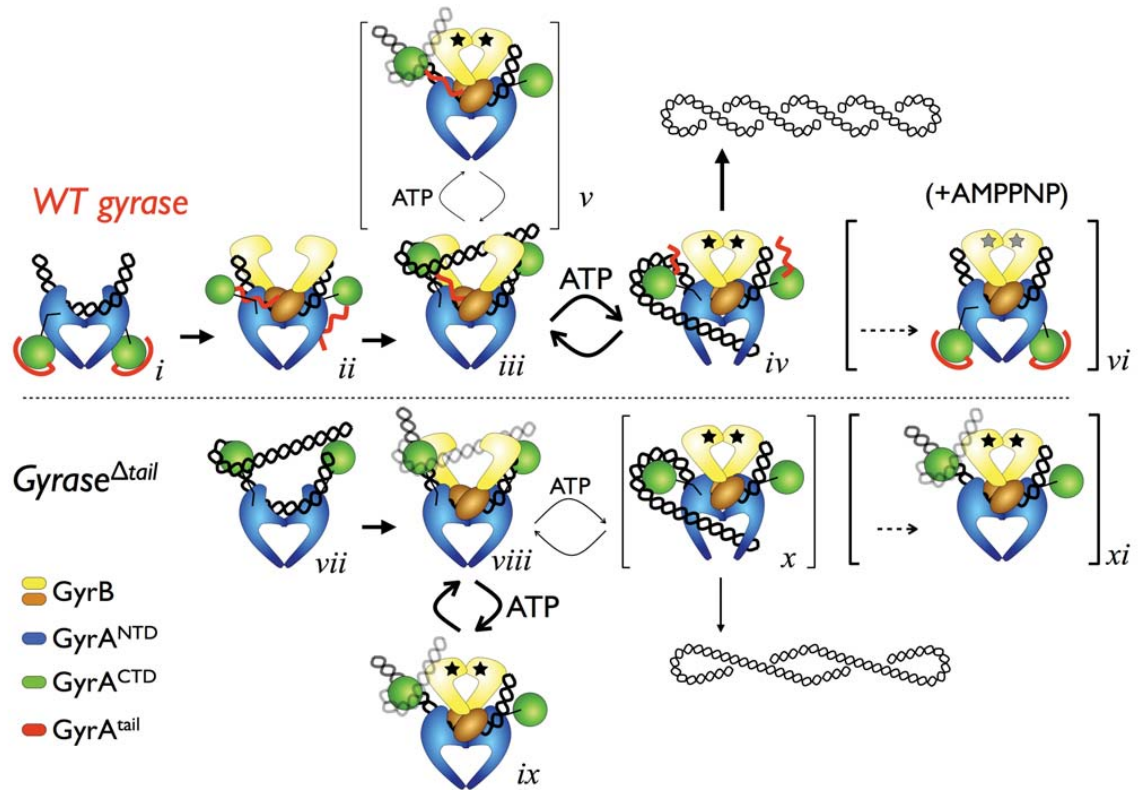


Figure 3.8. Model accounting for the action of the CTD tail on *E. coli* gyrase. Arrow thickness indicates the qualitative probability of transitioning between states. *i)* Full-length GyrA cannot wrap DNA on account of the repressive action of the tail on CTD activity and/or localization. *ii) & iii)* Binding of GyrB releases the repressive activity, either allosterically or by binding the tail directly, allowing wrapping. *iv)* ATP binding induces strand passage, freeing the tail from the action of GyrB; hydrolysis allows reset back to state (*iii*). Successive rounds of turnover allow for DNA supercoiling, which is extensive due to the tight coupling of CTD wrapping with ATPase status. *v)* Futile cycle in which ATP binding is accidentally not converted to a strand passage event, which is possibly due to incomplete wrapping. In wild-type gyrase, this event is rare (Sugino and Cozzarelli 1980; Bates, O'Dea et al. 1996) but may become more prevalent as DNA becomes more negatively supercoiled. *vi)* AMP-PNP permanently relieves the effect of GyrB on the tail, allowing re-engagement of the CTD to prohibit wrapping. Note that our data neither support nor exclude the possibility that a similar, tail-repressed CTD state might occur during normal enzyme cycling. *vii)* Removal of the tail frees the CTD to wrap DNA in the context of GyrA alone. *viii)* GyrB does not potentiate, and may even hinder, wrapping in the context of the holoenzyme. *ix)* Futile cycles of ATPase activity occur with increased frequency on account of the loss of coupling between CTD activity and GyrB status. *x)* Occasional ATPase cycles result in strand passage. The degree of supercoiling obtained without the tail is not as extensive as in wild-type gyrase, either because the inefficiency of the enzyme permits a relaxation "back-reaction" to occur (i.e., tail-less gyrase acts like a non-supercoiling type II topoisomerase), or because the ability of the enzyme to maintain a wrapped DNA state cannot compensate as its "load" is increased (i.e., the wrap on the CTD slips as DNA

becomes more supercoiled). At present, we cannot distinguish between these two possibilities. *xi*) AMPPNP has no effect on wrapping by a tail-less enzyme because the CTD is no longer under the control of GyrB.

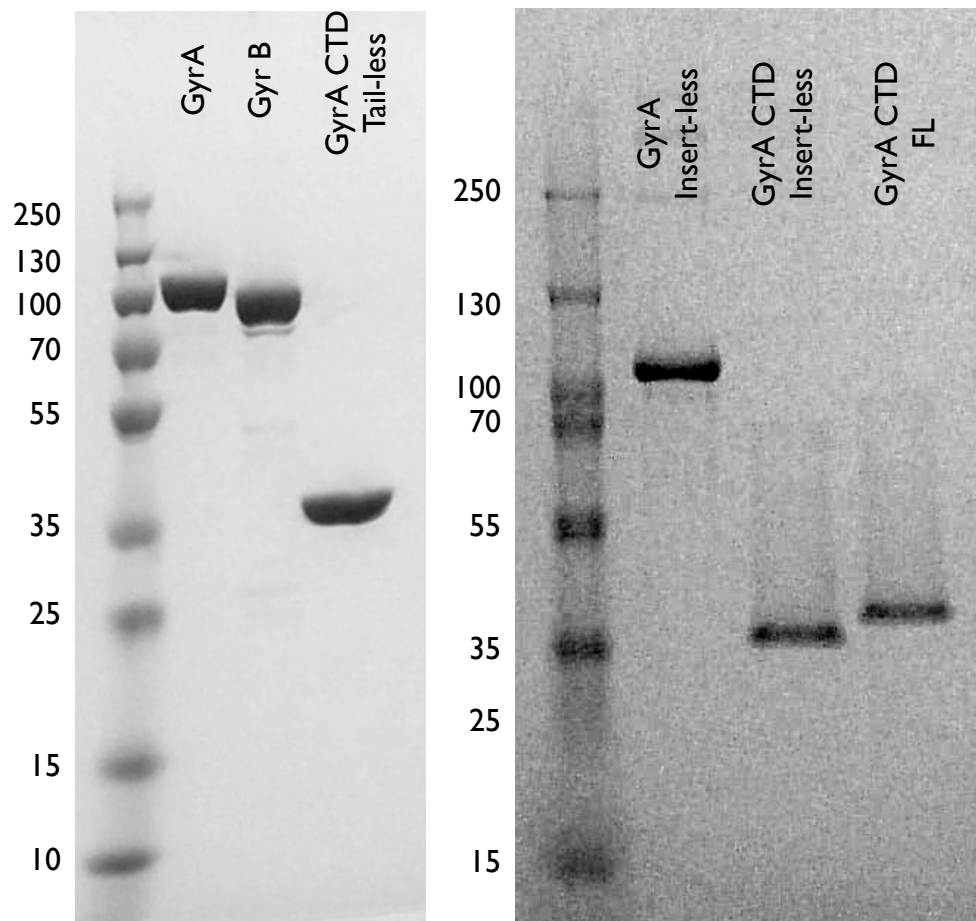


Figure S3.1. SDS-PAGE gels of purified *E. coli* gyrase proteins. Standard protein ladder labeled in kilodaltons.

Chapter 4 – Mechanisms for defining the supercoiling setpoint of DNA gyrase orthologs. Part II. The shape of the GyrA CTD is not a sole determinant for controlling supercoiling efficiency

(Portions reproduced from: Tretter, E.M. and Berger, J.M. (2012). J Biol Chem In Press)

Introduction

DNA topoisomerases are essential enzymes that can overwind, underwind, and disentangle double-helical DNA segments to maintain the topological state of chromosomes. Nearly all bacteria utilize a unique type II topoisomerase, gyrase, which actively adds negative supercoils to chromosomes using an ATP-dependent DNA strand passage mechanism; however, the specific activities of these enzymes can vary markedly from species to species. *E. coli* gyrase is known to favor supercoiling over decatenation (Peng and Marians 1993), whereas the opposite has been reported for *Mycobacterium tuberculosis* (*Mtb*) gyrase (Aubry, Fisher et al. 2006). Here, we set out to understand the molecular basis for these differences using structural and biochemical approaches.

Contrary to expectations based on phylogenetic inferences (Hsieh, Yen et al. 2010), we find that the dedicated DNA wrapping domains (the CTDs) of both gyrases are highly similar, both architecturally and in their ability to introduce writhe into DNA. However, the *Mtb* enzyme lacks a C-terminal control element recently uncovered in *E. coli* gyrase (see Chapter 3), and turns over ATP at a much slower rate. Together, these findings demonstrate that CTD shape is not the sole regulatory determinant of gyrase activity, and instead indicate that an inability to tightly couple DNA wrapping to ATP turnover is why *Mtb* gyrase cannot supercoil DNA to the same extent as its γ -proteobacterial counterpart. Our observations demonstrate that gyrase has been modified in multiple ways throughout evolution to fine-tune its specific catalytic properties.

Results and Discussion

Structure of the *Mtb* GyrA CTD

To first assess the physical basis for the activity differences seen between *Mtb* and *E. coli* gyrase, we determined the structure of the *Mtb* GyrA CTD. Crystals of the isolated domain (residues 514-838) were obtained in the spacegroup P2₁2₁2₁ and diffracted to 1.65 Å resolution. The structure was solved by molecular replacement using a poly-serine model of the *E. coli* GyrA CTD (residues 532-853, PDB 1ZIO) (**Figure 4.1A**) (Ruthenburg, Graybosch et al. 2005). The final model, which comprises amino acids 514-538 and 547-819 of the *Mtb* CTD, was refined to an R_{work}/R_{free} of 18.76/22.21% with excellent stereochemistry (**Table 1**).

As seen in other GyrA and ParC CTDs, the *Mtb* gyrase CTD forms a “ β -pinwheel” structure similar to, but topologically distinct from, canonical β -propellers (**Figure 4.1B**). The *Mtb* CTD structure turns out to closely mimic that of the *E. coli* CTD (1.45 Å rmsd), and likewise displays a positively-charged band around the disc's

perimeter that is thought to assist with DNA binding and/or bending (**Figure 4.1C**) (Corbett, Shultzaberger et al. 2004). As with the *E. coli* CTD, the GyrA box element of the *Mtb* domain also is disordered. Interestingly, the *Mtb* CTD also adopts a distinctly non-planar conformation, despite lacking a moderately-conserved proline that has been suggested to be promote the formation of the spiral state adopted by the GyrA CTDs of *E. coli* and *Xanthomonas campestris* (Hsieh, Yen et al. 2010) (**Figure 4.1D**); this finding indicates that the existence of a proline at this position is not responsible for configuring the CTD into a particular shape. Overall, inspection of the *Mtb* CTD structure failed to highlight any obvious structural features that might have accounted for the differences in activity reported for *Mtb* and *E. coli* gyrase.

DNA binding and wrapping by the *Mtb* CTD and *Mtb* gyrase

Because biochemical properties are not necessarily evident from structure alone, we next asked if the *Mtb* and *E. coli* CTDs might associate with DNA differently. We first compared the relative affinities of two CTDs for short DNA substrates using fluorescence polarization. In this assay, a fluorescently-labeled, 37 basepair duplex oligonucleotide was incubated with varying amounts of purified *E. coli* or *Mtb* gyrase CTD. In the course of conducting these studies, we noted that the *E. coli* CTD constructs used for biochemical and structural studies by other groups actually employed a variant that lacked some or all of a non-conserved acidic tail located at the C-terminus of the domain (Ruthenburg, Graybosch et al. 2005). These constructs bound DNA robustly in our hands, whereas a full-length CTD bearing an intact tail failed to bind DNA at all (see Chapter 3). By contrast, the full-length *Mtb* CTD bound DNA with an affinity similar to that of the tail-truncated *E. coli* domain ($K_{d, app} = 80 \text{ nM} \pm 16 \text{ nM}$ and $90 \text{ nM} \pm 21 \text{ nM}$, respectively) (**Figure 4.2**). An inspection of GyrA amino acid sequences shows that the *Mtb* CTD naturally lacks ~15 residues from its C-terminal tail compared to *E. coli* (see **Figure 3.1B**). Thus, while the innate DNA binding properties of the core CTD are similar between *E. coli* and *Mtb* gyrase, their CTD tails and the regulatory capacities of these elements are distinct.

DNA wrapping by the CTD is essential for supporting supercoiling by gyrase (Kampranis and Maxwell 1996). Reasoning that binding affinity might not necessarily imply an equal proclivity to introduce writhe into DNA, we set out compare the wrapping potential of the *Mtb* and *E. coli* CTDs using “topology footprinting” (Reece and Maxwell 1991; Ruthenburg, Graybosch et al. 2005). In this assay, each CTD was first individually mixed with nicked plasmid DNA, after which the DNA was ligated, trapping any writhe constrained by the protein. Bound CTD molecules were removed by SDS/proteinase K, and the supercoiling state of the DNA resolved by native agarose gel electrophoresis. Inspection of the topoisomers resulting from this treatment shows that both the full-length *Mtb* and tail-truncated *E. coli* CTDs were able to constrain a similar amount of writhe with respect to one another (**Figure 4.3A**). Consistent with its binding defect, the full-length *E. coli* CTD did not show any ability to wrap DNA (see Chapter 3).

Because the *E. coli* gyrase holoenzyme (Rau, Gellert et al. 1987), but not the isolated GyrA subunit (Reece and Maxwell 1991), can constrain writhe, we were curious if *Mtb* gyrase and its GyrA subunit, which naturally lack a portion of the *E. coli* CTD tail, would behave similarly. When assayed by topology footprinting, *Mtb* GyrA was able to wrap DNA to a similar degree as its isolated CTD, whereas *E. coli* GyrA failed to exhibit

any wrapping (**Figure 4.3B**). By contrast, both the *E. coli* and *Mtb* holoenzymes wrapped DNA nearly equivalently (**Figure 4.3C**). To probe these differences further, we next assessed if DNA wrapping by *Mtb* gyrase could be blocked by the non-hydrolyzable ATP analog AMP-PNP, a behavior seen for *E. coli* gyrase (Kampranis, Bates et al. 1999; Heddle, Mittelheiser et al. 2004). Strikingly, the ability of *Mtb* gyrase to introduce writhe was unaffected by the presence of AMP-PNP, whereas wrapping by *E. coli* gyrase was all but abolished (**Figure 4.3D**). Taken together, these results demonstrate that the extent of DNA wrapping induced by *E. coli* and *Mtb* gyrase is not appreciably distinct; rather, only *E. coli* gyrase appears capable of regulating wrapping in an ATP-dependent manner.

Decatenation by *Mtb* gyrase

Having established that an ability to switch between wrapped and unwrapped states, rather than an inherent structural or biochemical feature of the GyrA CTD, is point of divergence for the *Mtb* and *E. coli* orthologs, we turned our attention to other properties of the two enzymes. We first examined DNA decatenation, which has been reported to be more efficient with *Mtb* gyrase than with the *E. coli* protein (Aubry, Fisher et al. 2006). Decatenation was quantified by incubating each reconstituted holoenzyme with kinetoplast DNA (kDNA) and ATP, and then resolving the products using native agarose-gel electrophoresis. The large, catenated network of DNA minicircles that comprises kDNA is unable to enter the gel unless individual minicircles are liberated by decatenation by gyrase.

Contrary to our expectations, we found that the observed decatenation efficiency of *E. coli* gyrase was comparable to, or even slightly better than, that seen for the *Mtb* enzyme (**Figure 4.4**). Although the basis for this discrepancy was not immediately apparent, it did not arise from the presence of a significant portion of inactive enzyme in our *Mtb* gyrase preparations. Indeed, the specific activities of both our gyrase preparations were sufficient to nearly fully supercoil a target plasmid at a >50-fold molar excess of substrate DNA over enzyme under standard published conditions (**Fig. 4.5A, Methods**). This level of activity, which may arise as a consequence of a more extensive purification procedure than that used in prior studies, is ~5-10-fold greater than previously observed for the *Mtb* enzyme.

DNA supercoiling by *Mtb* gyrase

If *Mtb* and *E. coli* gyrase decatenate DNA similarly, then why might *E. coli* require a topo IV for chromosome segregation, whereas *M. tuberculosis* does not? To address this question, we re-examined the DNA supercoiling activity of the two proteins in detail. One clue came from inspection of our kDNA decatenation data (**Figure 4.4**), which indicated that the minicircles liberated by *E. coli* gyrase were supercoiled more readily than those produced by the *Mtb* enzyme. Since kDNA is an unusual substrate, we further compared the activities of both gyrases on relaxed circular DNA in presence of ATP.

Analysis of the resultant products by agarose-gel electrophoresis at first appeared to show comparable supercoiling activities between the two orthologs (**Figure 4.5A**). However, we noticed that the band containing the supercoiled DNA species produced by *Mtb* gyrase often appeared somewhat “fuzzy,” or less condensed. Because native gels

have an innate limit on their ability to resolve topoisomers that are significantly underwound with respect to one another (Clark and Leblanc 2009), we turned to chloroquine gels to determine if the degree of DNA supercoiling introduced by *Mtb* gyrase might differ from that of *E. coli*. Chloroquine is a weak intercalating agent that separates supercoiled topoisomers and allows for the resolution of negatively supercoiled DNA bands that would otherwise run together as a single high-mobility band on a native gel (Clark and Leblanc 2009). A portion of each reaction used in our native-gel studies was run on an agarose gel in the presence of 3 µg/ml chloroquine, after which the chloroquine was soaked out, and replaced with ethidium bromide for visualization. Notably, this analysis revealed that, at comparable concentrations, *Mtb* gyrase is unable to introduce as many negative supercoils into DNA as *E. coli* gyrase (**Figure 4.5B**).

Although this experiment suggested that *Mtb* and *E. coli* gyrase differ in yet another fundamental property, it did not rule out the possibility that strand passage by the *Mtb* enzyme might simply be slow, and hence failed to go completion during the course of our assay (30 min). To assess this possibility, we assayed supercoiling over a range of times. The reconstituted *E. coli* and *Mtb* gyrase enzymes were added to relaxed plasmid DNA and ATP at two concentrations (5 and 20 nM), both of which completely supercoiled the substrate in our enzyme titration experiments, and incubated at 37° C for 1, 5, 10, 30, and 90 min. Time points that exceeded 30 minutes were supplemented with additional ATP to prevent ATP-independent relaxation events from occurring as the nucleotide cofactor was depleted, and the results again visualized using agarose gel electrophoresis in the absence and presence of 3 µg/ml chloroquine. We found that supercoiling for both enzymes is complete within 30 min, and that even at longer times, *Mtb* gyrase still does not underwind DNA to the same degree as *E. coli* (**Figure 4.6**).

ATPase activity of *Mtb* gyrase

Because *Mtb* gyrase is able to robustly bind and wrap DNA, but lacks a CTD control element (the tail) that permits the coordination between nucleotide turnover and strand passage in its *E. coli* counterpart, we hypothesized that the slower and less extensive supercoiling seen for this mycobacterial enzyme might arise from a similar coupling inefficiency. To test this idea, we examined the ATPase activity of *Mtb* and *E. coli* gyrase using a malachite green colorimetric assay in which ATP hydrolysis is measured by phosphate release (Lanzetta, Alvarez et al. 1979). We conducted these measurements by incubating reconstituted gyrase in our supercoiling assay conditions with salmon sperm DNA, which is known to dramatically stimulate the ATPase activity of the enzyme (Maxwell and Gellert 1984). The reactions were incubated for different amounts of time using a fixed starting concentration of DNA and ATP. Surprisingly, we found that while both orthologs exhibited ATPase activity, *Mtb* gyrase turned over ATP at a significantly lower rate than *E. coli* gyrase (**Figure 4.7**). Taken together, these data suggest that strand passage is closely linked to ATP hydrolysis in *Mtb* gyrase, but that two factors – a lack of coupling between DNA wrapping and ATP binding, as well as an intrinsically slow rate of ATP turnover – contribute to the holoenzyme’s reduced supercoiling capacity.

Conclusion

In this chapter, we set out to structurally and biochemically define which elements and characteristics of *Mtb* gyrase might differ from its well-studied counterpart in *E. coli*. We were particularly intrigued as to whether the CTD of *Mtb* GyrA might play a role, since this domain has been implicated in the controlling the specificity of the gyrase and topo IV strand passage mechanism in numerous contexts (Kampranis and Maxwell 1996; Corbett, Schoeffler et al. 2005).

We began examining these issues by first determining the structure of the *Mtb* GyrA CTD. Phylogenetic and structural analyses had suggested that a buried proline (Pro636 in *E. coli* GyrA) imparts a non-planar, spiral shape to the domain in certain CTDs (Hsieh, Yen et al. 2010); this “lockwasher” configuration, when present, has been suggested to enhance DNA wrapping and supercoiling by the gyrase holoenzyme (Ruthenburg, Graybosch et al. 2005). As the *Mtb* CTD lacks the proline, we reasoned that the domain might adopt a more flattened conformation that would impair DNA wrapping, and thereby the lower the supercoiling propensity of *Mtb* gyrase as a whole. Instead, we found that the *Mtb* CTD was highly similar structurally to that of *E. coli* GyrA (**Figure 4.1**), and that the domain could robustly bind and introduce writhe into DNA (**Figures 4.2, 4.3**). Thus, the difference in shape between relatively planar CTDs (as with *B. burgdorferi* GyrA) (Corbett, Shultzaberger et al. 2004) and more skewed forms is not due to solely to a single amino acid. Likewise, the *Mtb* CTD did not contain a particularly distinct shape that might have been responsible for the altered supercoiling functions exhibited by *Mtb* gyrase.

The emergence of this result led us to probe the specific activities of *Mtb* gyrase further. Comparative biochemical studies with isolated domains and subunits, as well as the reconstituted holoenzyme, eventually highlighted several points of divergence between *Mtb* and *E. coli* gyrase. One was an ability of full-length *Mtb* GyrA and GyrA CTD to wrap DNA (**Figure 4.3**). This activity is exhibited by mutants of *E. coli* GyrA that lack a non-conserved C-terminal tail (see Chapter 3), but not by the full-length *E. coli* proteins (Reece and Maxwell 1991). A second difference was that *Mtb* gyrase failed to show any coupling between DNA wrapping and nucleotide binding (**Figure 4.3**), an established property of the *E. coli* enzyme (Hedde, Mitelheiser et al. 2004). A third variance was the finding that *Mtb* gyrase does not possess an enhanced ability *per se* to decatenate DNA relative to *E. coli*, but rather exhibits a diminished capacity to underwind DNA to same extent (**Figures 4.4-4.6**). Finally, we found that *Mtb* gyrase hydrolyzes ATP at a much slower rate than *E. coli* (**Figure 4.7**).

Together, these distinguishing features have important implications for the mechanism of both *Mtb* gyrase and gyrases in general (**Figure 4.8**). The ability of gyrase to supercoil DNA derives in part from its DNA wrapping activity (Liu and Wang 1978; Kampranis and Maxwell 1996), which juxtaposes two DNA segments *in cis* prior to strand transport. This property in turn disfavors the passing of two distal DNA segments through each other, impeding DNA decatenation and unknotting. *Mtb* gyrase can wrap DNA efficiently, thereby setting the stage for a single round of supercoiling. However, it does not appear to link the formation or dissolution of this wrapped state to the ATPase cycle (**Figure 4.3**), which in *E. coli* gyrase occurs through the action of the tail appended to the C-terminus of GyrA (see Chapter 3). Indeed, removal of the GyrA

tail reduces the supercoiling setpoint of *E. coli* gyrase significantly, to a level close to that observed here for *Mtb* gyrase, yet does not alter the innate decatenation properties of the enzyme (**Figure S4.2**); these findings suggest that part of the reason *Mtb* gyrase does not possess as robust a supercoiling capacity as *E. coli* is in part because it lacks this evolutionary modification. By failing to stabilize or release a wrapped DNA state at the correct point in time, the *Mtb* enzyme may be more prone to “slippage”, particularly as more energy becomes required to supercoil DNA to higher and higher levels. This characteristic would in turn favor strand passage events that do not have as great an energetic consequence, such as those needed to support chromosome disentanglement.

However, an inability to connect DNA wrapping to ATP binding, or to supercoil DNA extensively, does not imply that *Mtb* gyrase is an inefficient enzyme. In *E. coli*, removal of the GyrA tail dramatically slows the rate of supercoiling, but has little effect on the ATPase activity of gyrase as whole, resulting in a large increase of apparently futile cycles. By contrast, *Mtb* gyrase hydrolyzes ATP at a much lower rate, but also is slow to supercoil DNA (**Figures 4.6, 4.7**). Thus, ATP turnover rates and strand passage appear to be linked in *Mtb* gyrase, even though the enzyme lacks the tail found in *E. coli* GyrA. Why *Mtb* gyrase is slow is not clear, but conceivably could be an adaptation to the sluggish life cycle of its host organism (23 h average generation time) (O'Sullivan, McHugh et al. 2010).

Materials and Methods

Protein purification

The coding regions of the *M. tuberculosis* GyrA CTD (514-838), full-length *M. tuberculosis* *gyrA* (1-838), and full-length *gyrB* (1-714) were amplified from genomic DNA (ATCC) and cloned into a derivative of pET28b behind an N-terminal, tobacco etch virus (TEV) protease-cleavable hexahistidine tag using an in-house ligation independent cloning vector system (pLIC). The truncated *E. coli* GyrA CTD (531-853) and full-length *E. coli* *gyrA* (1-875) and *gyrB* (1-804) genes were cloned into pET28b. Proteins were expressed in *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene) by inducing log-phase cells with 0.25 mM isopropyl- β -D-thiogalactopyranoside either for 4 h at 37°C, or overnight at 18°C. Full-length *M. tuberculosis* GyrB was expressed in BL21-Codon Plus (DE3)-pLysS cells by inducing log-phase cells with 1mM isopropyl- β -D-thiogalactopyranoside for 3 h at 30°C. Cells were harvested by centrifugation, resuspended in 20 mM Tris-HCl pH 7.9, 800 mM NaCl, 30 mM imidazole, 10% glycerol, and protease inhibitors (1 μ M leupeptin, 1 μ M pepstatin A, and 1 mM phenylmethylsulfonyl fluoride), and frozen dropwise in liquid nitrogen for storage at -80°C.

For purification, cells were sonicated and centrifuged, and the clarified lysate was passed over a Ni²⁺ affinity column (Amersham Biosciences). His-tagged protein was eluted with 20mM Tris-HCl pH 7.9, 100 mM NaCl, 500 mM imidazole, 10% glycerol, and protease inhibitors (1 μ M leupeptin, 1 μ M pepstatin A, and 1 mM phenylmethylsulfonyl fluoride), concentrated and exchanged into the same buffer containing 30 mM imidazole, and then incubated overnight at 4°C with 1-1.5 mg hexahistidine-tagged TEV protease (Kapust, Tozser et al. 2001). Following TEV cleavage, the mixture was passed over a Ni²⁺ affinity column, and the flow-through

collected, concentrated (Millipore Amicon Ultra-10/30), and run over an S-200 or S-300 gel filtration column (Amersham Biosciences) in 50 mM Tris-HCl pH 7.9, 500 mM KCl, 10% glycerol, and 2mM 2-mercaptoethanol. Peak fractions were pooled and concentrated by centrifugal filtration (Millipore Amicon Ultra-10/30), and assessed for purity by SDS-PAGE (**Figure S4.1**). *M. tuberculosis* GyrB was purified using the above protocol but with the following Ni²⁺ affinity column buffers: load/wash buffer (20mM Tris-HCl 7.9, 500mM NaCl, 5mM imidazole) and elution buffer (20mM Tris-HCl 7.9, 500mM NaCl, 250mM imidazole).

Crystallization, data collection, and structure solution

Purified *M. tuberculosis* GyrA CTD at 12 mg/mL was dialyzed overnight at 4°C against 50mM NaCl and 10mM Tris-HCl pH 7.5. Crystals were grown in mosquito drop hanging drop format by mixing 100 nL of protein with 100 nL well solution containing 0.2 M Calcium acetate hydrate and 20% Polyethylene glycol 3,350. For harvesting, crystals were looped and flash-frozen in liquid nitrogen. A single native dataset was collected at Beamline 8.3.1 at the Advanced Light Source at Lawrence Berkeley National Laboratory. Native data were indexed and reduced as P2₁2₁2₁ using HKL2000 (**Table 1**) (Otwinowski Z 1997). Phasing by molecular replacement using the *E. coli* GyrA CTD (residues 532-853, PDB Code 1ZI0) revealed one *M. tuberculosis* GyrA CTD protomer per asymmetric unit (Ruthenburg, Graybosch et al. 2005; McCoy, Grosse-Kunstleve et al. 2007). Density modification and initial model building were performed by PHENIX (Adams, Grosse-Kunstleve et al. 2002); subsequent cycles of manual rebuilding and refinement were performed using COOT (Emsley and Cowtan 2004). TLS parameters were analyzed with the TLSMD server (Painter J 2006), and 15 TLS groups were introduced in the later stages of refinement. The final model was refined to 1.65 Å resolution, with a final R_{work}/ R_{free} of 22.2/18.7%. Molprobity analysis (Lovell, Davis et al. 2003) showed a total of 99% of residues in the most favored regions of Ramachandran space, with none in disallowed regions. The atomic structure and coordinate files have been deposited with the PDB, ID code 3UC1. Ribbon structures, electron density, and surface charge images were all prepared in PyMOL (DeLano 2002).

DNA binding assays

DNA binding was determined by fluorescence anisotropy. The DNA substrate for binding was a random, 37-base pair segment with 40% GC content. Annealed oligos 5'-TAA AGT CTA GAG ACA CGC ATA GTC AAT GAC GGA GTT A -3' and 5'-|56-FAM|TAA CTC CGT CAT TGA CTA TGC GTG TCT CTA GAC TTT A -3' where 56-FAM indicates the position of a carboxyfluorescein dye for visualization, were purchased from Integrated DNA Technologies and resuspended in TE Buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0). For binding, varying amounts of truncated *E. coli* GyrA CTD or *M. tuberculosis* GyrA CTD were incubated with 20nM of fluorescently labeled duplex oligonucleotide at room temperature in the dark in 20 mM Tris-HCl pH 7.5, 70 mM KCl, 10% glycerol, and 1 mM MgCl₂. Fluorescence anisotropy measurements were performed using a Victor 3V (Perkin Elmer) multi-label plate reader. Data points represent the average of three independent measurements on each plate, while error bars represent the standard deviation between measurements. Binding curves were fit to a simplified

version of the single site binding equation that holds for our experimental conditions using Kaleidagraph version 4.0 (Synergy software):

$$\theta = \frac{[L]}{K_{d,app} + [L]}$$

where θ represents the fraction of ligand binding sites filled, $K_{d,app}$ is the apparent dissociation constant, and L is the ligand concentration.

DNA relaxation, decatenation, and supercoiling assays

pSG483, a derivative of pUC19 containing a unique Nb.BbvCI nicking site (NEB), was used for supercoiled and relaxed DNA substrates. Negatively-supercoiled plasmid was purified from *E. coli* with a maxiprep kit (Macherey Nagel). Relaxed plasmid was made using nick-ligation by first nicking maxiprep plasmid DNA with Nb.BbvCI and then ligating with T4 DNA ligase. Kinetoplast DNA (kDNA) from *Crithidia fasciculata* was purified in house as described (Shapiro, Klein et al. 1999). DNA relaxation, decatenation, or supercoiling assays (30 μ L) were performed in a buffer containing 15 mM Tris-HCl pH 7.5, 13% glycerol, 6 mM MgCl₂, 0.1 mg/ml BSA, 70 mM KCl, 1 mM dithioerythritol, 2 mM ATP and 300 ng (6nM) DNA substrate. To assess activity, reactions were supplemented with varying amounts of reconstituted holoenzyme, ATP was added to reaction mixtures and reactions allowed to proceed for 30 minutes at 37 °C. Time course reactions contained 4 mM ATP, with an additional 2 mM ATP added at 60 minutes. Reactions were stopped by the addition of SDS (1% final concentration) and EDTA (10 mM final concentration). Quenched reactions were analyzed by electrophoresis through 1.0% agarose gels with 1x TAE running buffer and chloroquine gels contained 3 μ g/ml chloroquine (Sigma) in the gel and running buffer. Gels were run at 1.7-5.0 V/cm for 4-12 hours at 25°C, stained with ethidium bromide (EtBr), and visualized by UV trans-illumination.

Topological footprinting assays

DNA writhe assays (30 μ L) were performed in a buffer containing 15 mM Tris-HCl pH 7.5, 13% glycerol, 6 mM MgCl₂, 0.1 mg/ml BSA, 70 mM KCl, and 300 ng (6 nM) nicked pSG483 as DNA substrate. Varying amounts of CTD were added to reaction mixtures and the reactions equilibrated at 37°C for 20 min, after which 60 units of ligase and 1 mM ATP were added to each reaction mixture. Reactions were then allowed to incubate at 37°C for an additional 20 min. Reactions were stopped by the addition of SDS (1% final concentration), EDTA (10 mM final concentration), and proteinase K (50 μ g/ml final), and incubated at 37°C for 30 minutes. Reactions were analyzed by electrophoresis through 1.0% agarose gels with 1x TAE running buffer. Gels were run at 1.7 V/cm for 19-21 hours at 25°C, stained with ethidium bromide (EtBr), and visualized by UV trans-illumination.

To capture a gyrase holoenzyme “topology footprint”, we used an ATP-independent assay technique to prevent gyrase from supercoiling the plasmid DNA substrate. In these reactions, gyrase holoenzyme was incubated with relaxed plasmid and eukaryotic topoisomerase IB, which relaxes DNA supercoils in the absence of ATP (Champoux and Dulbecco 1972; Shuman 1998). Holoenzyme writhe assays were performed in the presence and absence of 2 mM AMP-PNP using relaxed pSG483

plasmid and topoisomerase IB purified in house from wheat germ (Dyan, Jendrisak et al. 1981).

ATPase assays

ATPase assays were performed using a malachite green/molybdate assay for free phosphate. Reactions, run under supercoiling assay conditions, contained 50 nM gyrase, 1 mM ATP, and 50 μ M sheared salmon-sperm DNA (Sigma). Reactions were initiated by the addition of enzyme and ATP and incubated for varying times at 37°C. After incubation, 200 μ l of malachite green reagent (0.034% malachite green, 10 mM ammonium molybdate, 1 N hydrochloric acid, 3.4% ethanol, and 0.01% Tween 20) was added to each reaction and incubated at room temperature in the dark for 5 minutes. The malachite green reagent was made fresh daily and sterile filtered. Absorbance at 620 nm was measured using a Perkin-Elmer Victor3V plate reader. All reactions were performed in triplicate.

Tables

Table 4.1. Data collection and refinement statistics

Data Collection	
Resolution (Å)	50-1.65
Wavelength (Å)	1.1000
Space Group	P2 ₁ 2 ₁ 2 ₁
Unit cell dimensions (a, b, c) (Å)	38.87, 82.84, 83.43
Unit cell angles (α, β, γ) (°)	90, 90, 90
I/σ (last shell)	27.1 (3.6) ^a
R _{merge} (last shell) (%) ^b	7.4 (5.6)
Completeness (last shell) (%)	100 (100)
Redundancy	7.1 (7.0)
Unique Reflections ^c	236445
Refinement	
Resolution (Å)	41.72-1.65
No. of reflections	33225
R _{work} (%) (last shell) ^d	18.76 (25.13)
R _{free} (%) (last shell) ^e	22.21 (28.18)
Structure and Stereochemistry	
No. atoms	2733
Protein	2309
Water	303
Acetate	1
Calcium	1
Glycerol	1
B factor (Å²)	
Protein	20.72
Water	29.02
r.m.s.d. bond lengths (Å)	0.009
r.m.s.d. bond angles (°)	1.182
Ramachandran Plot (%)^f	
Favored Region	99.0
Allowed Region	1.0
Outliers	0

^aThe values in parentheses indicate the highest resolution shell.

^b $R_{\text{merge}} = \sum_{\text{hkl}} I_i(\text{hkl}) / \sum_{\text{hkl}} I(\text{hkl})$, where $I_i(\text{hkl})$ is the intensity of an observation and $I(\text{hkl})$ is the mean value for its unique reflection. Summations cover all reflections.

^c $N_{\text{obs}}/N_{\text{unique}}$.

^d $R_{\text{work}} = \sum_{\text{hkl}} ||F_{\text{obs}}| - k|F_{\text{calc}}|| / \sum_{\text{hkl}} |F_{\text{obs}}|$

^e R_{free} was calculated same way as R_{work} , but with the reflections excluded from refinement. The R_{free} set was chosen using default parameters in PHENIX (Adams, Grosse-Kunstleve et al. 2002).

^fCategories were defined by Molprobit (Lovell, Davis et al. 2003).

Figures

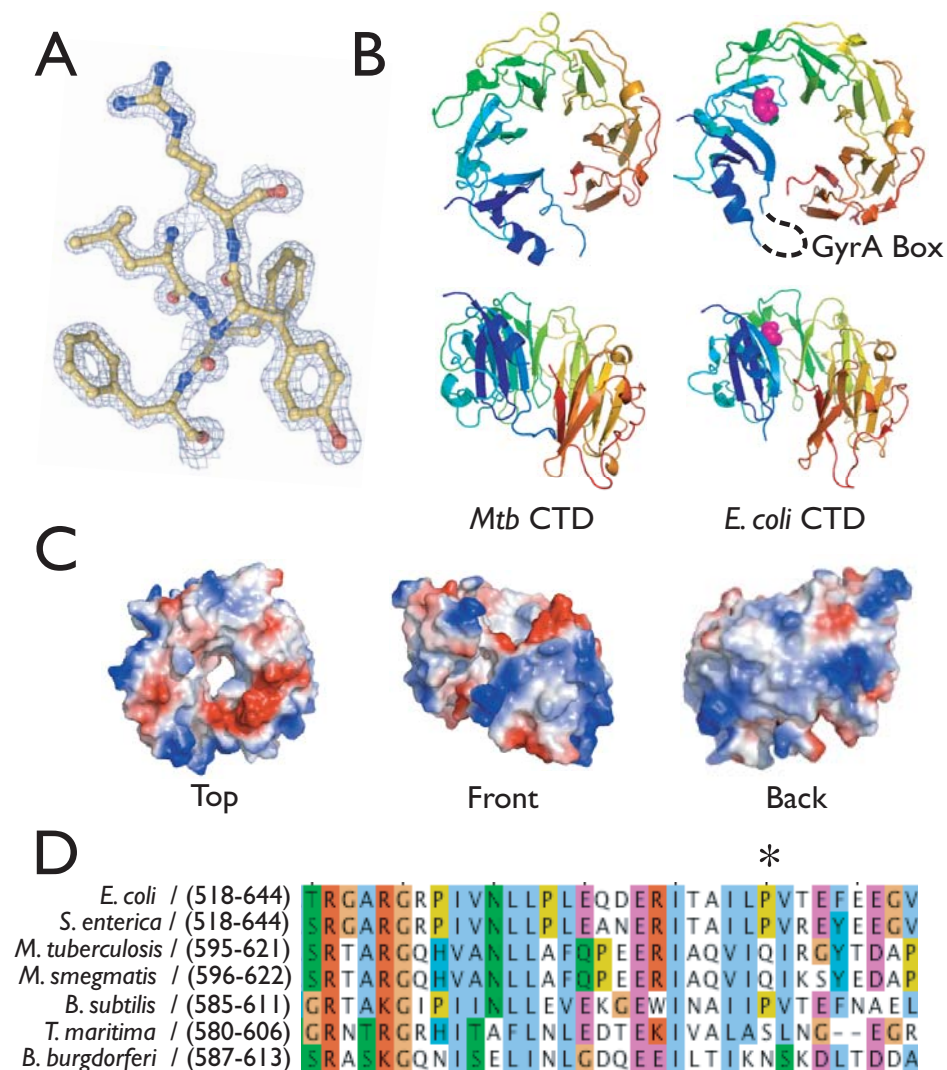


Figure 4.1. *M. tuberculosis* CTD structure. (A) Representative electron density from a refined $2F_o - F_c$ map contoured at 2σ . (B) Ribbon model of *Mtb* and *E. coli* GyrA CTD showing face-on (top panel) and front views (bottom panel). On the *E. coli* CTD model, the conserved proline is depicted in magenta spheres and the hypothetical location of the GyrA Box is indicated with a dashed black line. (C) Electrostatic surface representation of *Mtb* GyrA CTD. (D) Sequence alignment of representative CTDs that contain or lack the conserved proline (marked with an asterisk) that follows the GyrA Box.

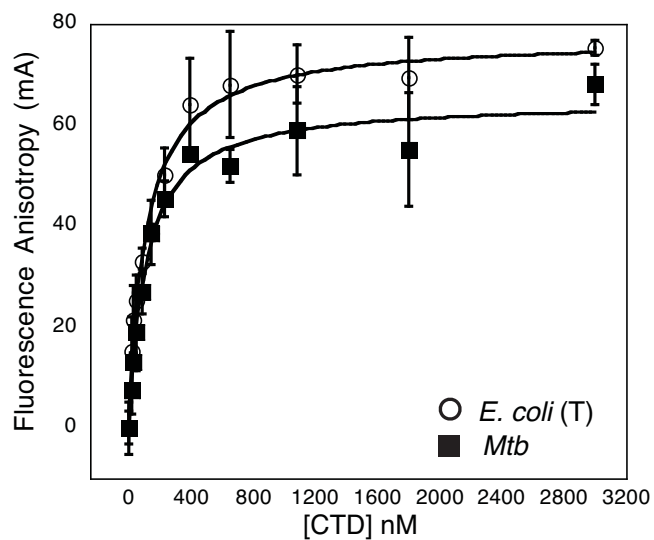


Figure 4.2. DNA binding by CTD constructs. Binding of fluorescein-tagged, double-stranded oligonucleotides (20 nM), monitored as a function of CTD concentration by fluorescence anisotropy. The apparent K_d values are as follows (truncated *E. coli* CTD $90 \text{ nM} \pm 21 \text{ nM}$, *Mtb* CTD $80 \text{ nM} \pm 16 \text{ nM}$). The truncated *E. coli* CTD is indicated by (T).

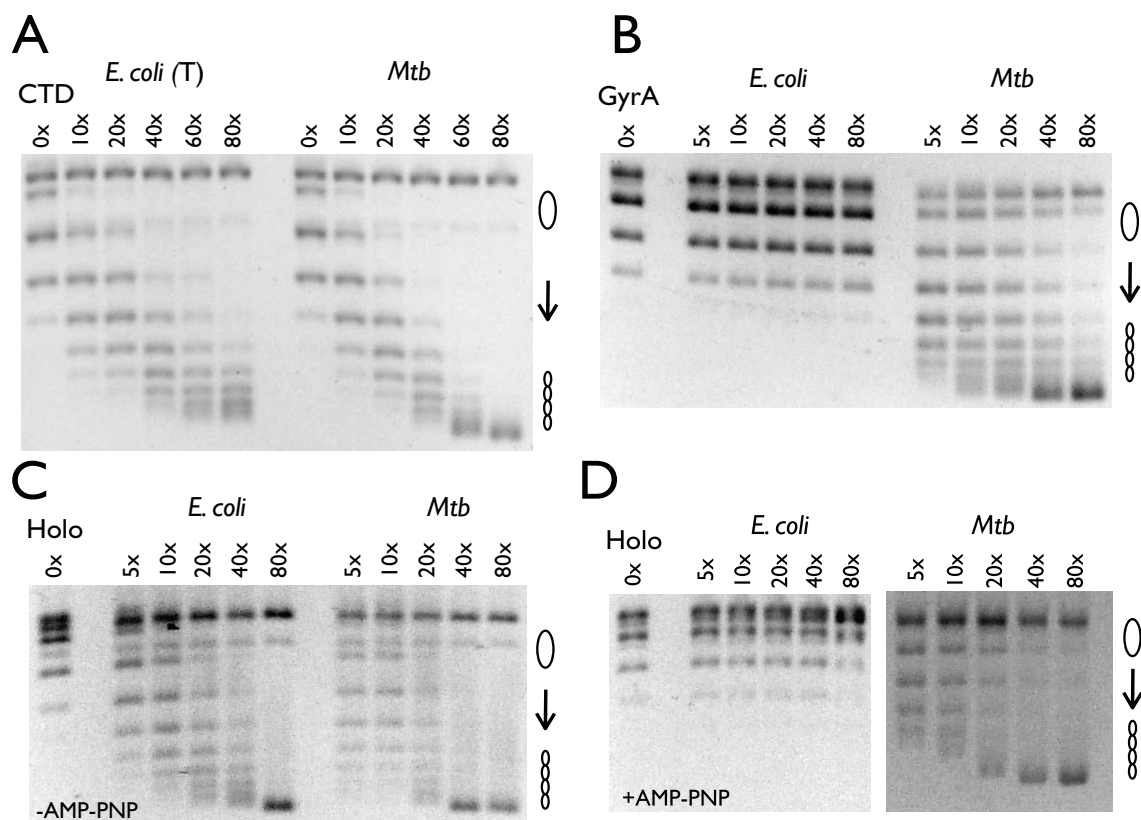


Figure 4.3. Topology footprint assays. (A) Nicked pSG483 (6 nM) was incubated with varying amounts of CTD and ligase (indicated as a molar excess of CTD over plasmid). The truncated *E. coli* CTD is indicated by (T). (B) Nicked pSG483 (6 nM) was incubated with varying amounts of GyrA dimer and ligase (indicated as a molar excess of CTD over plasmid). (C and D) Relaxed pSG483 (6 nM) was incubated with varying amounts of reconstituted holoenzyme and topo IB in the absence (C) and presence (D) of AMP-PNP (2 mM) (indicated as a molar excess of CTD over plasmid). The positions of relaxed and supercoiled DNA species are labeled with cartoon representations on the right.

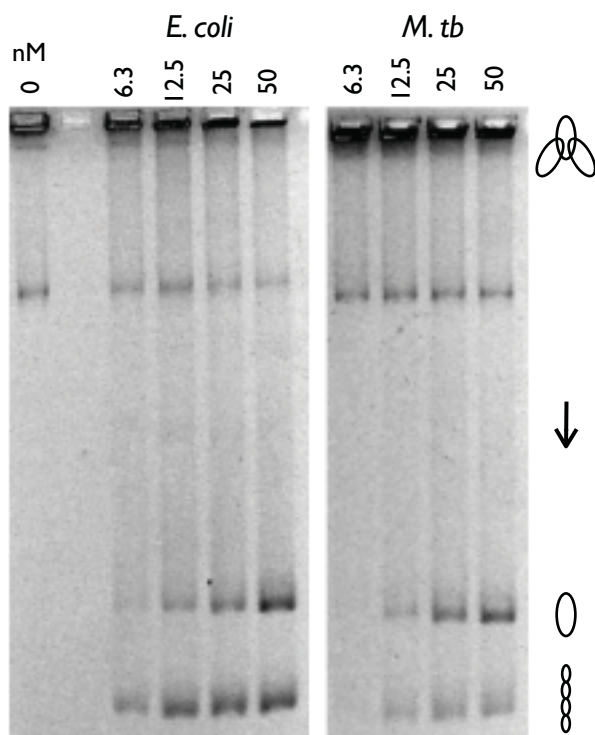


Figure 4.4. *E. coli* and *M. tuberculosis* holoenzyme decatenation assay.
The positions of the kinetoplast network, relaxed released circles, and supercoiled released circles are labeled with cartoon representations on the right.

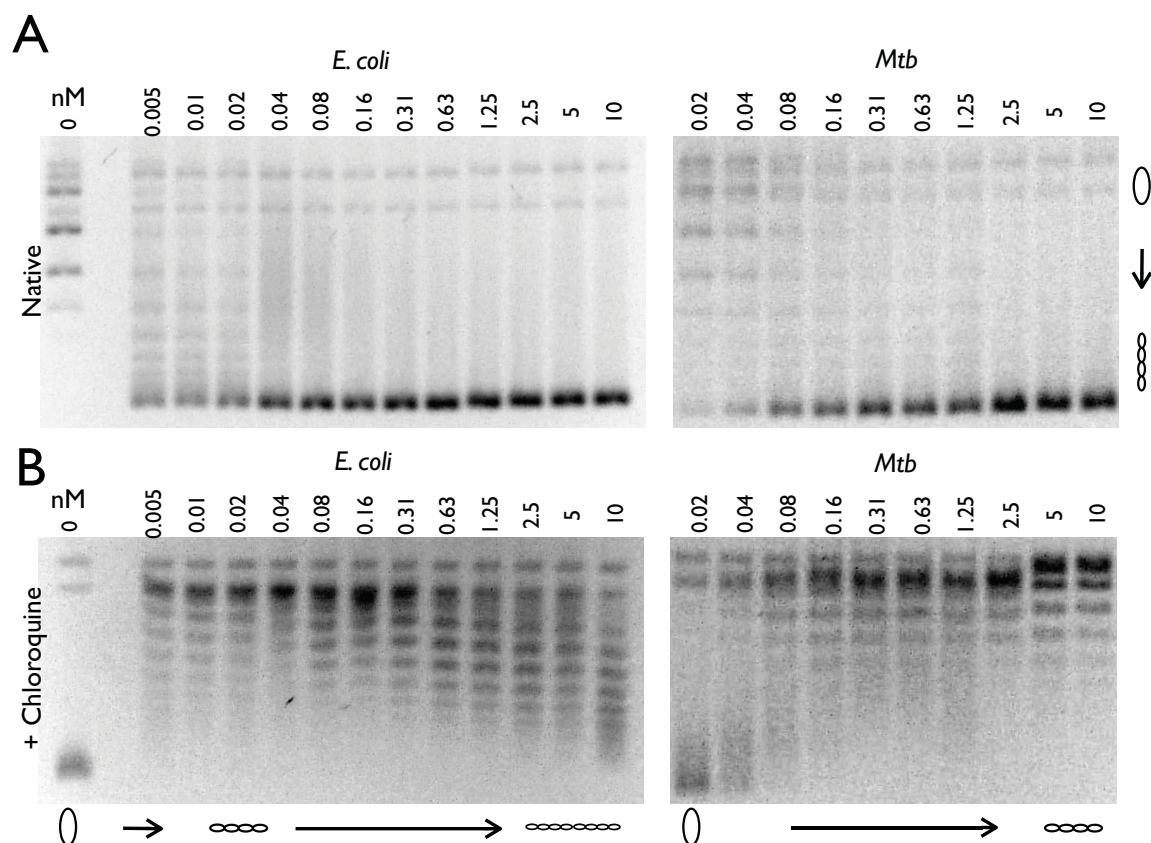


Figure 4.5. *E. coli* and *M. tuberculosis* negative supercoiling holoenzyme assays. A portion of each sample was run on a 1% agarose gel in the absence (A) and presence (B) of 3 $\mu\text{g/mL}$ chloroquine. Protein concentrations are listed in nM holoenzyme and DNA topoisomers are labeled with cartoon representations across the bottom of the chloroquine gel. Relaxed and supercoiled DNA species are labeled with cartoon representations on the right of the native gel.

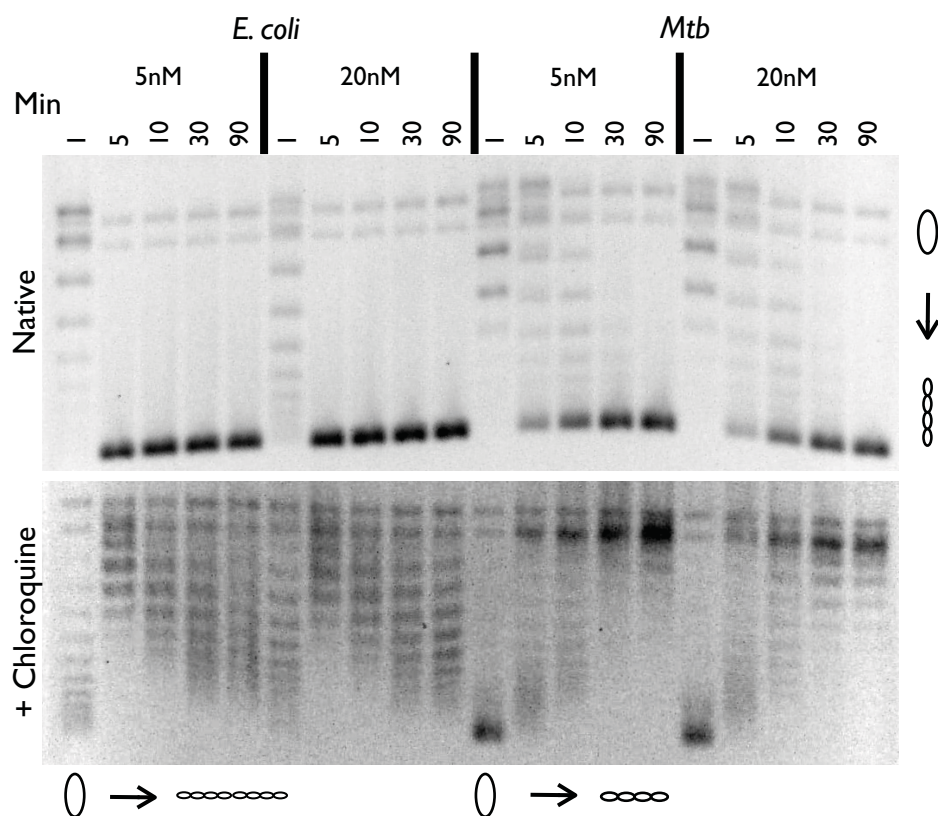


Figure 4.6. Negative supercoiling time course assay using 5 nM and 20 nM holoenzyme. Time points are listed in minutes. A portion of each sample was run on a 1% agarose gel in the absence (García-Estrada, Prada et al.) and presence (bottom) of 3 µg/mL chloroquine. Protein concentrations are listed in nM holoenzyme and DNA topoisomers are labeled with cartoon representations across the bottom of the chloroquine gel. Relaxed and supercoiled DNA species are labeled with cartoon representations on the right of the native gel.

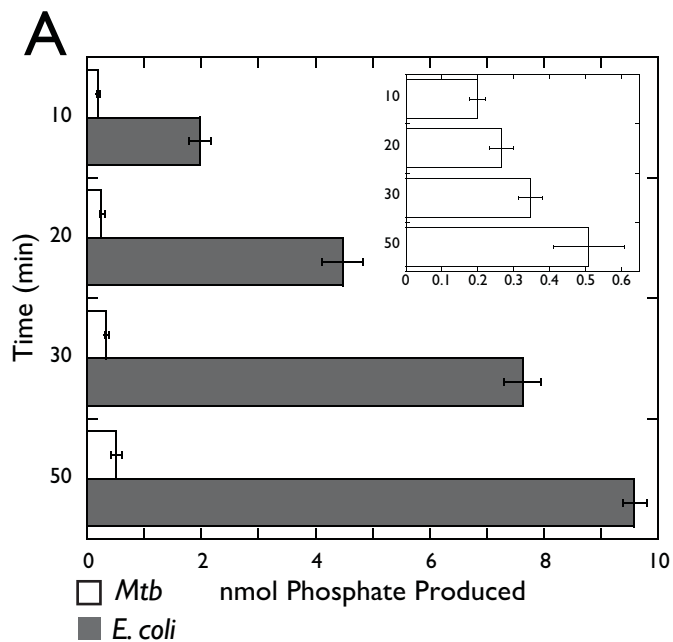


Figure 4.7. ATPase activity. *E. coli* and *M. tuberculosis* gyrase (A) ATPase activities are plotted as a function of nmol Phosphate produced (x-axis) and time in minutes (y-axis). Inset graph provides magnified version of *M. tuberculosis* gyrase ATPase data.

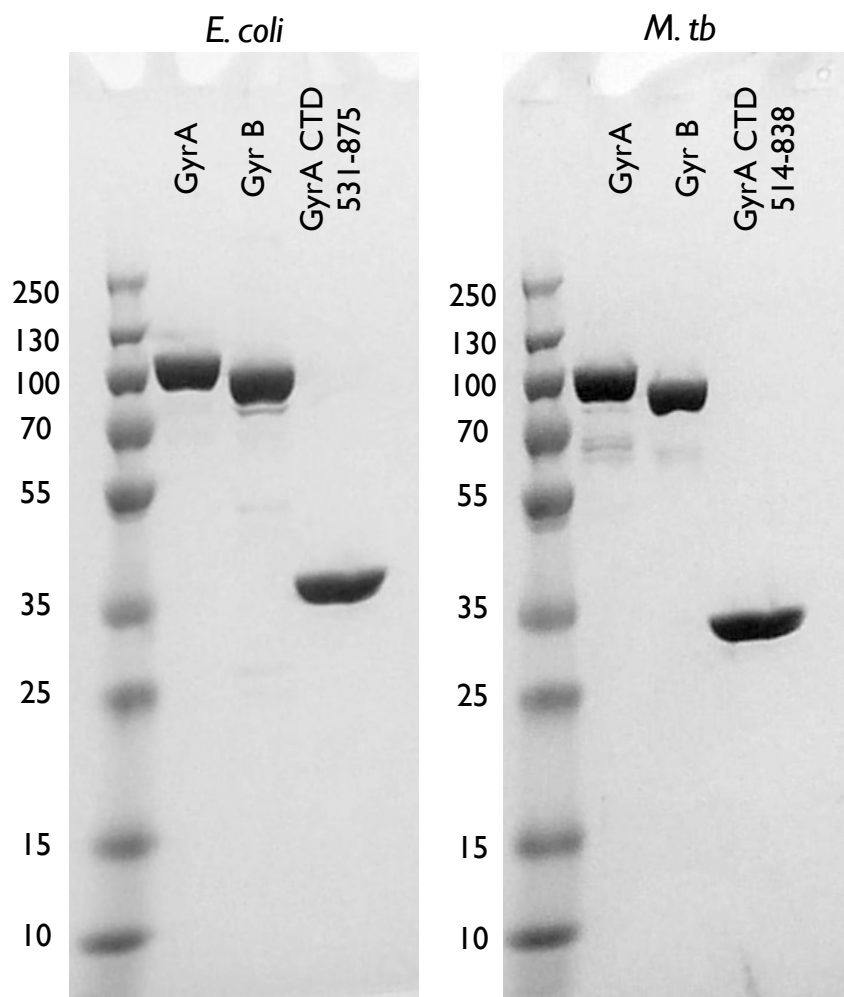


Figure S4.1. SDS-PAGE gels of purified *Mtb* and *E. coli* gyrase proteins.
Standard protein ladder labeled in kilodaltons.

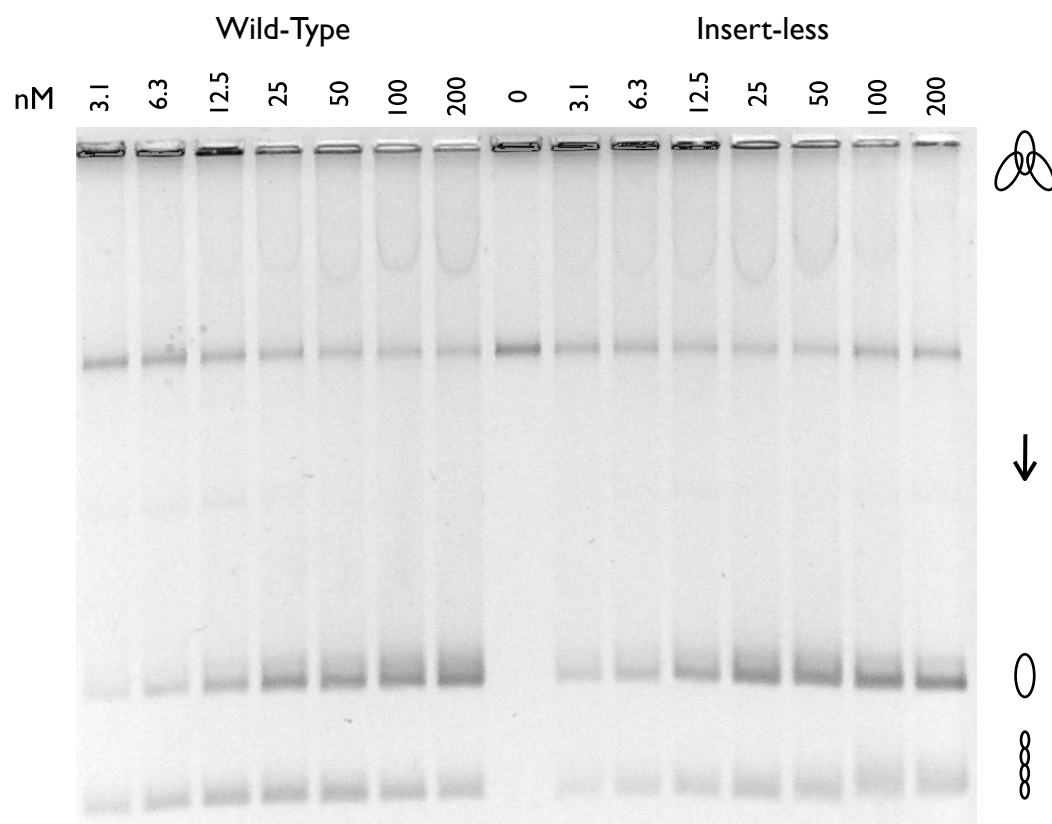


Figure S4.2. Wild-Type and Insert-less *E. coli* holoenzyme decatenation assay. The positions of the kinetoplast network, relaxed released circles, and supercoiled released circles are labeled with cartoon representations on the right.

Chapter 5 – Evolution and Specialization of the *A. aeolicus* type IIA DNA Topoisomerase

(Portions reproduced from: Tretter, E.M., Lerman, J.C., and Berger, J.M. (2009). Proc Natl Acad Sci 107, 22055-22059)

Introduction

Cellular DNA topology is managed by topoisomerases, molecular machines that both regulate supercoiling and unlink tangled chromosomes (Schoeffler and Berger 2008). The bacterial/archaeal type IIA topoisomerase superfamily, which includes DNA gyrase and topoisomerase IV (topo IV), couples ATP binding and hydrolysis with DNA binding, cleavage, and strand transport to physically move one duplex through another (Gellert, Mizuuchi et al. 1976; Kato, Nishimura et al. 1990) (**Figure 5.1A**). Gyrase and topo IV share significant structural homology and are thought to be paralogs which resulted from gene duplication of an ancestral gyrase protein (Huang 1996; Forterre, Gribaldo et al. 2007).

Both gyrase and topo IV are A₂B₂ heterotetramers. Their functional differences are thought to be predicated upon a C-terminal DNA binding domain (the CTD) that is appended to the homologous A protomers (*gyrA* and *parC*) (**Figure 5.1A**) (Morrison and Cozzarelli 1979; Liu, Rowe et al. 1983; Kampranis and Maxwell 1996; Corbett, Schoeffler et al. 2005). The gyrase CTD is believed to constrain a positive supercoil by bending DNA around its surface, allowing the enzyme to introduce a negative supercoil upon strand passage (Reece and Maxwell 1991; Corbett, Shultzaberger et al. 2004; Ruthenburg, Graybosch et al. 2005). Loss of the GyrA CTD, or the GyrA box, a conserved motif found in the first blade of the GyrA CTD (**Figure 5.1B**), eliminates gyrase's ability to negatively supercoil DNA (Kampranis and Maxwell 1996; Ward and Newton 1997; Kramlinger and Hiasa 2006). The topo IV CTD has been proposed to control enzyme activity not by wrapping DNA around its surface, but by helping to capture a DNA segment *in trans* for strand passage (Corbett, Schoeffler et al. 2005). Removal of the topo IV CTD ablates the holoenzyme's preference for its favored substrates, positively supercoiled and catenated DNAs (Stone, Bryant et al. 2003; Corbett, Schoeffler et al. 2005).

This dissertation aims to understand how specific evolutionary modifications to the CTD, such as blade number and CTD shape, help fine-tune the substrate selectivity and functional output of prokaryotic type IIA topoisomerases. In this chapter we investigate one particularly intriguing, and uncharacterized, type IIA topoisomerase derives from *Aquifex aeolicus*, a member of one of the most thermophilic and deeply branched families within the bacterial domain (Deckert, Warren et al. 1998). Determination of the *A. aeolicus* genome sequence led to the annotation of this enzyme as a DNA gyrase (Deckert, Warren et al. 1998), a conclusion supported by subsequent phylogenetic comparisons, which found that the B subunit aligns with *gyrB* orthologs (Guipaud and Forterre 2001; Forterre, Gribaldo et al. 2007). However, these later studies also noted that the *A. aeolicus* CTD lacked a canonical GyrA-box motif within its putative *gyrA* gene, suggesting that this open reading frame might in fact code for ParC,

a subunit of topo IV (Guipaud and Forterre 2001; Forterre, Gribaldo et al. 2007). This dichotomy was further accentuated by: 1) the lack of either a clear ParE homolog, or a GyrA subunit containing an appropriate GyrA-box in *A. aeolicus*, and 2) the present understanding that all bacterial species are believed to minimally retain and require DNA gyrase for viability (Forterre, Gribaldo et al. 2007; Forterre and Gabelle 2009). These observations not only raised a basic question as to whether the *A. aeolicus* enzyme functions as a gyrase or topo IV, but also suggested that inspection of the *A. quifex* system could afford a new general perspective into the evolution of bacterial type IIA topoisomerases.

To address these issues, we carried out structural and biochemical analyses of the sole type II topoisomerase of *A. aeolicus*. We first determined the crystal structure of the *A. aeolicus* GyrA/ParC CTD to 1.3 Å resolution, and characterized the ability of the domain to introduce writhe into DNA. We then purified the holoenzyme and characterized its ability of to relax, supercoil, or decatenate DNA. We find that the *A. aeolicus* CTD bears physical hallmarks of both GyrA and ParC CTDs, but, like topo IV, it is unable to alter DNA writhe. Similarly, the activity profiles of the *A. aeolicus* holoenzyme follow those exhibited by topo IV, and not gyrase. However, subunit-mixing experiments show that the *A. aeolicus* GyrB subunit can support DNA supercoiling with *E. coli* GyrA, but not DNA relaxation with *E. coli* ParC. Moreover, replacing the *A. aeolicus* CTD with the CTD from *T. maritima* DNA gyrase bestowed a negative supercoiling activity on the *A. aeolicus* holoenzyme. These findings, together with bioinformatic analyses, indicate that *A. aeolicus* originally possessed a DNA gyrase that later converted directly into an enzyme with topo IV-like properties. Our findings provide the first demonstration that bacterial species can exist without gyrase, and further highlight a new path for the evolution of functionally distinct bacterial type IIA topoisomerases.

Results and Discussion

Structure of GyrA C-terminal domain

To assess the functionality of the *A. aeolicus* type IIA topoisomerase, we first examined the structure of the C-terminal domain (CTD) of the GyrA subunit. Crystals of isolated *A. aeolicus* GyrA CTD (residues 489-769) were obtained in the spacegroup P1 and diffracted to 1.3 Å, the highest resolution yet reported for any topoisomerase region. The structure was solved using multiple isomorphous replacement. Interpretable density was present for amino acids 494-769 for each of the three *A. aeolicus* GyrA CTD chains present in the asymmetric unit (**Figure 5.2A**). The final model was refined to an $R_{\text{work}}/R_{\text{free}}$ of 17.46/19.20% and shows excellent stereochemistry (**Table 5.1**).

As seen in other GyrA and ParC CTDs, the *A. aeolicus* CTD forms a planar disc comprising a multi-bladed “β-pinwheel” structure similar to, but topologically distinct from, canonical β-propellers (**Figure 5.2B**) (Corbett, Shultzaberger et al. 2004). Analogous to other GyrA/ParC CTDs, the *A. aeolicus* CTD retains a positively charged band around the disc’s perimeter that is thought to assist with DNA binding and/or bending (**Figure S5.1A**) (Corbett, Shultzaberger et al. 2004; Hsieh, Farh et al. 2004). The *A. aeolicus* CTD most closely superposes with the CTD of *B. burdorferi* GyrA (1.48 Å rmsd), although the *A. aeolicus* CTD adopts a partially open configuration seen in

other CTD structures such as *E. coli* GyrA and *B. stearotherophilus* ParC (**Figure S5.2B**) (Hsieh, Farh et al. 2004; Ruthenburg, Graybosch et al. 2005). Comparison of the *A. aeolicus* CTD with the *X. campestris* GyrA CTD reveals that this open configuration is due to the loss of the GyrA-box in the *A. aeolicus* domain, which in *X. campestris*, closes off the pinwheel by pairing the first blade with the sixth (**Figure 5.2C**) (Hsieh, Yen et al. 2010). *X. campestris* was used in this comparison as it is the only structure to date which includes a complete GyrA-box. By comparison, the *E. coli* ParC CTD also lacks the GyrA box loop (as well as the entire sixth blade), forming a “C”-shaped structure that is even more open than that seen for the *A. aeolicus* CTD (3.01 Å rmsd).

***Aquifex aeolicus* holoenzyme activity**

Because the *A. aeolicus* CTD structure contained hallmarks of both GyrA and ParC CTDs, we next sought to determine how the intact topoisomerase acts on different DNA substrates. To accomplish this, we prepared and reconstituted the heterotetrameric holoenzyme. Because *A. aeolicus* is a hyperthermophile, we initially screened for activity over a range of temperatures (37° to 90° C). Although activity was detected across this range, optimal activity was observed at 70° C.

The first reaction we tested was negative supercoiling, the defining hallmark of gyrase (Gellert, Mizuuchi et al. 1976). In this reaction, relaxed circular DNA is incubated with enzyme and ATP, after which the reaction products are resolved by agarose-gel electrophoresis. Significantly, the *A. aeolicus* enzyme failed to produce any sign of negatively-supercoiled DNA from the starting substrate, even when present at a >30-fold molar excess (**Figure 5.3A**). This result provided the first clues that, contrary to its annotated status, the type II topoisomerase present in *A. aeolicus* is not a gyrase.

We next assessed the ability of the enzyme to catalyze DNA relaxation. In the presence of ATP, *A. aeolicus* DNA gyrase was able to remove both negative and positive DNA supercoils. Interestingly, positive supercoil relaxation was more robust than the relaxation of negative supercoils (~5-fold), a characteristic of topo IV enzymes (**Figure 5.3B, C**) (Crisona, Strick et al. 2000; Corbett, Schoeffler et al. 2005). The ability to relax negative supercoils in the presence of ATP directly conflicts with the canonical activity of DNA gyrase. Together, these data demonstrate that, at least functionally, the *A. aeolicus* type II topoisomerase serves as a topo IV.

To further test this claim, we examined DNA decatenation, the known specialty of topo IV. Activity was quantified by incubating the reconstituted enzyme with kinetoplast DNA (kDNA) and ATP, and the reaction again resolved by electrophoresis. As a network of catenated DNA minicircles, intact kDNA is too large to enter the gel, whereas the small minicircles can be liberated by the *A. aeolicus* topoisomerase and detected. The observed decatenation activity of the *A. aeolicus* topoisomerase was similar in efficiency to the relaxation activities of the enzyme, although not as robust as seen for positive supercoil relaxation (**Figure 5.3D**). Moreover, the released minicircles showed no sign of supercoiling, a reaction known to be catalyzed by gyrase in this assay (Kramlinger and Hiasa 2006).

DNA writhe introduction by the *A. aeolicus* ParC C-terminal domain

The distinguishing ability of gyrase to negatively supercoil DNA relies on the DNA wrapping properties of the GyrA CTD (Kampranis and Maxwell 1996). These properties can be observed experimentally and quantitated by a “topology footprint” (Reece and Maxwell 1991; Ruthenburg, Graybosch et al. 2005). In this type of experiment, the isolated CTD is mixed with relaxed plasmid DNA, after which a type IB topoisomerase (e.g., eukaryotic topo I) is added to remove compensatory supercoils formed by CTD binding and wrapping. The subsequent stripping of bound CTD protomers by SDS/proteinase K leaves the DNA with an “afterimage” of the number of supercoils originally sequestered by the CTDs; the assay also reports on the handedness of the wrap, which is known to be positive for both gyrase and the isolated CTD fold (Liu and Wang 1978; Reece and Maxwell 1991). Examination of the *A. aeolicus* CTD by this method showed it was only marginally capable of introducing writhe into DNA, to a similar degree as the *E. coli* ParC CTD (**Figure S5.3A**). By contrast, control experiments using the *E. coli* GyrA CTD show robust positive supercoiling of the DNA, consistent with its known chiral wrapping properties. Together with the activities seen for the holoenzyme, this result further supports the proposition that the type IIA topoisomerase genes of *A. aeolicus* code for a functional topoIV enzyme.

The type II topoisomerase of *A. aeolicus* is a gyrase/topo IV hybrid

Although the reaction profiles of the *A. aeolicus* enzyme are clearly distinct from those seen for a gyrase, they did not immediately comport with phylogenetic analyses of the protein placing the type II topoisomerase in the gyrase clade (Forterre, Gribaldo et al. 2007). To examine this discrepancy in more detail, we constructed alignments using 46 representative bacterial GyrA/ParC and 49 GyrB/ParE protein sequences. Consistent with prior annotations (Deckert, Warren et al. 1998; Guipaud and Forterre 2001; Forterre, Gribaldo et al. 2007), our neighbor-joining trees placed the complete *A. aeolicus* A subunit with ParC sequences and the intact B subunit with GyrB sequences (**Figure S5.5, S5.6**). However, when we separated and aligned the individual N- and C-terminal domains of the *A. aeolicus* A subunit with other gyrase/topo IV sequences, only the CTD fell within the ParC clade (**Figure S5.5**). By contrast, the NTD, which houses the active site for DNA cleavage, aligned with known GyrA proteins. Moreover, the placement of the CTD within the type II topoisomerase phylogenetic tree resides close to the branch point that separates the gyrase/topo IV clades. Thus, while *A. aeolicus*’s type IIA topoisomerase behaves functionally like a topo IV, its ancestry at the sequence level is overall more similar to DNA gyrase.

***A. aeolicus* GyrB supports negative supercoiling activity**

The discrepancy between sequence and function for the *A. aeolicus* type II topoisomerase suggested a direct means of typing its paternity. Specifically, if the *A. aeolicus* enzyme was truly of gyrase heritage, we reasoned that its individual subunits might work with those of *E. coli* gyrase. Conversely, if it descended from topo IV, then functional chimeras might be formed from *E. coli* topo IV. Such subunit mixing experiments have been shown to work for very distantly related gyrases, such as those found in chloroplasts (Wall, Mitchenall et al. 2004).

To test this idea, we reconstituted A₂B₂ heterotetramers using *E. coli* GyrA and *A. aeolicus* GyrB, and measured their ability to negatively supercoil relaxed DNA in the presence of ATP. As a control, *E. coli* GyrA also was mixed with *E. coli* ParE, the topo IV paralog of GyrB. As expected, the *E. coli* GyrA/ParE mixture failed to support supercoiling (**Figure S5.3B**). By contrast, *A. aeolicus* GyrB functionally paired with *E. coli* GyrA to negatively supercoil the substrate (**Figure 5.4A**), with an optimal activity observed between 37°-47° C. Moreover, the addition of *A. aeolicus* GyrB to *E. coli* ParC did not give rise either to supercoiling or to DNA relaxation (**Figure S5.3C**). These data support the concept that, despite its biochemical functions, the *A. aeolicus* enzyme is of direct gyrase ancestry.

***A. aeolicus*: *T. maritima* GyrA fusion supports negative supercoiling activity**

Because the *A. aeolicus* GyrB subunit is able to support negative supercoiling activity and the CTD of the GyrA subunit was the only portion of the holoenzyme to align as a topo IV, we next asked whether the *A. aeolicus* GyrA CTD could be swapped with a *bone fide* GyrA CTD to restore gyrase function. Using the GyrA CTD from the thermophile *Thermotoga maritima*, which contains a canonical GyrA-box (Figure S5.1), we constructed a chimera with the *A. aeolicus* GyrA/ParC NTD. We then reconstituted A₂B₂ heterotetramers using the chimera and *A. aeolicus* GyrB and measured this enzyme's ability to negatively supercoil relaxed DNA in the presence of ATP. Agarose gel electrophoresis of the products shows that the *T. maritima* CTD was sufficient to bestow negatively supercoiling activity on the *A. aeolicus* holoenzyme (Figure 5.4B). Taken with our prior observations, these results further suggest the *A. aeolicus* enzyme is of direct gyrase ancestry.

Conclusion

Aquifex aeolicus possesses only one type II topoisomerase. Based on phylogenetic analyses, this enzyme was classified principally as gyrase (Deckert, Warren et al. 1998); however, the A subunit also lacked defining signatures for the GyrA box, a CTD motif required for gyrase activity (Ward and Newton 1997; Guipaud and Forterre 2001). This odd confluence of characteristics prompted us to investigate the *A. aeolicus* enzyme further. Through structural methods, we first found that the *A. aeolicus* A-subunit CTD displayed hallmarks of both gyrase and topoIV CTDs (**Figure 5.2**). We next biochemically characterized the purified *A. aeolicus* holoenzyme and found it to behave as a topo IV, with the ability to both decatenate kDNA and relax both positive and negative DNA supercoils (**Figure 5.3**). Significantly, the protein showed no propensity to negatively supercoil DNA, the defining property of gyrase. Moreover, like that of topo IV, the isolated *A. aeolicus* CTD proved unable to introduce writhe into a relaxed DNA plasmid (**Figure S5.3A**). These findings firmly establish the *A. aeolicus* type II topoisomerase as a functional topo IV, and distinguish this organism as the first bacterium found to lack gyrase functionality.

Despite these biochemical properties, the *A. aeolicus* protein nonetheless shows a generally closer kinship to gyrase at the amino acid sequence level (**Figure S5.5, S5.6**) (Guipaud and Forterre 2001; Forterre, Gribaldo et al. 2007). In addition, subunit-mixing experiments demonstrate that *A. aeolicus* GyrB can functionally pair with *E. coli* GyrA

subunit, but not *E. coli* ParC (**Figure 5.4, S5.3C**). The only portion of this protein to align with topo IV enzymes is the CTD; however, this element also maps close to the GyrA/ParC branch point, and swapping the *A. aeolicus* CTD with the CTD from *T. maritima* DNA gyrase furnishes the *A. aeolicus* holoenzyme with the ability to negatively supercoil relaxed DNA. These observations suggest that the functional conversion of a DNA gyrase into a topo IV can require relatively modest modifications of CTD, such as loss of the C-terminal domain GyrA box (Forterre, Gribaldo et al. 2007; Forterre and Gadelle 2009). Consistent with this idea, removal of the CTD or ablation of the GyrA box in *E. coli* GyrA eliminates negative supercoiling activity, but retains DNA relaxation and decatenation activities (Kampranis and Maxwell 1996; Kramlinger and Hiasa 2006).

Together, these lines of evidence suggest a model for how gyrase and topo IV arose evolutionarily (**Figure 5.5**). The ancestral protein likely was first formed as a consequence of a domain fusion between the CTD and topoisomerase core (Todd, Orengo et al. 1999; Chaudhuri, Soding et al. 2008). Because the CTD is itself composed of several repeats of a single subdomain, each of which contains remnants of the GyrA box motif present in the first blade, it is likely that this element descended from the duplication of a simple, ancestral greek-key type of β -sheet (Schoeffler and Berger 2005). The role of the CTD prior to its fusion with a topoisomerase A subunit event is unclear, as thus far the domain has been found only in gyrases and topo IVs; however, its ability to wrap DNA when the GyrA box is intact suggests that it may have originally acted as a type of nucleoid-associated protein (NAP) that could assist in DNA compaction (Dillon and Dorman 2010). The bacterium *Borrelia burgdorferi* is interesting in this regard, in that the CTD region of its *gyrA* gene is expressed independently and can form higher-order nucleoprotein complexes with DNA (Knight and Samuels 1999). If this initial pathway is correct, then the first CTD-modified enzyme to have appeared in bacteria would have been a gyrase. Once in place, a gene duplication event of *gyrB* and *gyrA*, followed by divergence of the CTD, would result in the relative specialization of topo IV from gyrase (Corbett, Schoeffler et al. 2005; Forterre, Gribaldo et al. 2007; Forterre and Gadelle 2009).

The type IIA topoisomerase of *A. aeolicus* is significant in this context, in that the overall phylogenetic relationships of its constituent subunits are consistent with the “gyrase-first” hypothesis. The enzyme also is noteworthy in that it suggests an alternative means for evolving a topo IV, namely, one that derives from direct modification of the *gyrA* gene. This “shortcut” to producing a topo IV-like function is more parsimonious than a gene duplication mechanism, in that the cell need not undergo multiple rounds of subunit gain, loss and fusion. The consequence of this approach is that *A. aeolicus* appears to have sacrificed its gyrase to gain topo IV. At present it is unclear why this organism has gone this route, although it is interesting to note that *A. aeolicus* is the most thermophilic bacteria identified to date, and it contains two copies of reverse gyrase, an enzyme that adds positive supercoils to DNA. As negatively-supercoiled DNA is more prone to thermal denaturation than overwound DNA, *A. aeolicus* may have inactivated its gyrase as a consequence of adaptation to ultra-high temperatures. This situation may be analogous to that seen in archaea, whose most thermophilic species also lack gyrase, and instead universally retain a specialized type IIB topoisomerase known as topo VI (Forterre, Bergerat et al. 1996; Bergerat, de Massy et al. 1997).

Materials and Methods

Protein purification

The coding region for residues 489-769 of *A. aeolicus* ParC CTD was amplified from genomic DNA (ATCC) and cloned into a derivative of pET28b using an in-house ligation independent cloning vector system, pLIC, behind an N-terminal, tobacco etch virus (TEV) protease-cleavable hexahistidine tag. The full-length *A. aeolicus* *gyrA* (1-769), *gyrB* (1-792), and *A. aeolicus gyrA* NTD:*T. maritima gyrA* CTD chimera (NTD:1-483 and CTD:482-804) genes were cloned into the same vector and the *E. coli* GyrA CTD (531-853) and ParC CTD (497-752) were cloned into pET28b. Proteins were expressed in *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene) by inducing log-phase cells with 0.5 mM isopropyl- β -D-thiogalactopyranoside for 4 h at 37°C. Full length *A. aeolicus* ParC was expressed similarly in BL21-Codon Plus (DE3)-PlysS cells. Cells were harvested by centrifugation, resuspended in 20 mM Tris-HCl pH 7.9, 800 mM NaCl, 30 mM imidazole, 10% glycerol, and protease inhibitors, and were frozen dropwise in liquid nitrogen. The *E. coli* GyrA CTD was expressed using auto-induction, where cells were brought to log phase at 37°C, grown overnight at 25°C, and harvested as described above (Studier 2005).

For purification, cells were sonicated and centrifuged, and the clarified lysate was passed over a Ni²⁺ affinity column (Amersham Biosciences). His-tagged protein was eluted with 20mM Tris-HCl pH 7.9, 100 mM NaCl, 500 mM imidazole, 10% glycerol, and protease inhibitors (1 μ M leupeptin, 1 μ M pepstatin A, and 1 mM phenylmethylsulfonyl fluoride), concentrated and exchanged into the same buffer containing 30 mM imidazole, and then incubated for 4 h at room temperature with hexahistidine-tagged TEV protease (Kapust, Tozser et al. 2001). Following TEV cleavage, the mixture was passed over a Ni²⁺ affinity column, and the flow-through was collected, concentrated, and run over an S-200 or S-300 gel filtration column (Amersham Biosciences) in 50 mM Tris-HCl pH 7.9, 500 mM KCl, 10% glycerol, and 2mM 2-mercaptoethanol. Peak fractions were pooled and concentrated by centrifugal filtration (Millipore Centriprep-10/30).

Preliminary purifications of *A. aeolicus* GyrA yielded a close molecular weight doublet observable by SDS-PAGE gel. Inspection of the DNA sequence revealed a stretch of nine nucleotides (1,419-1,428) that code for three rare tRNAs in *E. coli*, and that could account for the smaller protein product were these codons to result in premature termination during translation of the transcript. Codon optimization of the region resulted in the near elimination of the doublet from subsequent purifications (**Figure S5.6**). These codons were also optimized in the *A. aeolicus*:*T. maritima* GyrA chimera.

Crystalization, data collection, and structure solution

Purified *A. aeolicus* ParC CTD at 10-15 mg/mL was dialyzed overnight at 4°C against 50mM NaCl and 10mM Tris-HCl pH 7.5. Crystals were grown in hanging drop format by mixing 1 μ L of protein with 1 μ L well solution containing 225 mM ammonium nitrate and 20% polyethylene glycol 3500. For harvesting, crystals were equilibrated into 25% glycerol in 5 minute, 5% steps, followed by looping and flash-freezing in liquid nitrogen. Prior to heavy atom soaks, crystals were exchanged into low salt (50 mM

sodium nitrate), after which 10 mM of heavy atom salts ($K_2Pt(CN)_4$, K_2PtCl_4 , and $NaWO_4$) were added and the crystals soaked overnight.

A single native dataset and three anomalous datasets were collected at Beamline 8.3.1 at the Advanced Light Source at Lawrence Berkeley National Laboratory. Native data were indexed and reduced as P1 using HKL2000 (**Table 5.1**) (Otwinowski Z 1997). Anomalous data were indexed and reduced as P1 using the CCP4 program SCALA (1994). Phasing by SOLVE revealed three *A. aeolicus* ParC CTD protomers per asymmetric unit (Terwilliger and Berendzen 1999). Density modification and initial model building were performed by PHENIX (Adams, Grosse-Kunstleve et al. 2002); subsequent cycles of manual rebuilding and refinement were performed using COOT (Emsley and Cowtan 2004). TLS parameters were analyzed with the TLSMD server, and 24 (8 per CTD) TLS groups were introduced in the later stages of refinement (Painter J 2006). The final model was refined to 1.30 Å resolution, with a final R_{work}/R_{free} of 17.5/19.2%. Molprobit analysis (Lovell, Davis et al. 2003) showed a total of 98.2% of residues in the most favored regions of Ramachandran space, with none in disallowed regions. The atomic structure and coordinate files have been deposited with the PDB, ID code 3NO0.

DNA relaxation, decatenation, and supercoiling assays

pUC19 was used for supercoiled and relaxed DNA substrates. Negatively-supercoiled plasmid was purified from *E. coli* with a maxiprep kit (Macherey-Nagel) and positively supercoiled DNA was made using *A. fulgidus* reverse gyrase (Rodriguez 2002). Relaxed plasmid was made using purified wheat germ topoisomerase I (Dynan, Jendrisak et al. 1981). Kinetoplast DNA (kDNA) from *Crithidia fasciculata* was purified in house as described (Shapiro, Klein et al. 1999).

DNA relaxation or decatenation assays (30 μ L) were performed in a buffer containing 30 mM KCl, 25 mM Tris-HCl pH 7.9, 15% glycerol, 0.1 mg/ml bovine serum albumin (BSA), 5 mM $MgCl_2$, 1 mM spermidine, 2 mM dithiothreitol (DTT), and 300 ng (6nM) DNA substrate. Varying amounts of reconstituted holoenzyme were added to reaction mixtures, and reactions were allowed to proceed for 30 minutes at either 70° C or 37° C, as indicated. Reactions were stopped by the addition of SDS (1% final concentration) and EDTA (10 mM final concentration). Stopped reactions were analyzed by electrophoresis through 1.0% agarose gels with 1x TAE running buffer. Gels were run at 1.7-3.8 V/cm for 4-12 hours, stained with ethidium bromide (EtBr), and visualized by UV trans-illumination.

Topological footprinting assays

Relaxed plasmid pUC19 was made using purified wheat germ topoisomerase I (Dynan, Jendrisak et al. 1981). DNA writhe assays (30 μ L) were performed in a buffer containing 20 mM Tris-HCl pH 7.9, 13% glycerol, 1 mM $MgCl_2$, 0.1 mg/ml BSA, 30 mM KCl, 30 mM NaCl, and 300 ng (6 nM) DNA substrate. Varying amounts of CTD were added to reaction mixtures and the reactions equilibrated at 25°C for 20 minutes, after which 300 ng topo I was added to each reaction mixture. Reactions were then allowed to incubate at 25°C for an additional 20 min. Reactions were stopped by the addition of SDS (1% final concentration) and EDTA (10 mM final concentration) and heated to 65°C. Stopped reactions were analyzed by electrophoresis through 1.0%

agarose gels with 1x TAE running buffer. Gels were run at 1.7 V/cm for 16 hours, stained with ethidium bromide (EtBr), and visualized by UV trans-illumination.

Sequence alignments and phylogenetic trees

Alignments of 45 full-length, C-terminal domains, and N-terminal domains of GyrA and ParC sequences and 49 full-length GyrB and ParE sequences were performed using MAFFT (**Table 5.2**) (Kato, Asimenos et al. 2009). Neighbor joining phylogenetic trees were created in Jalview and modified using Fig Tree (Rambaut 2009; Waterhouse, Procter et al. 2009).

Updated genome annotation and ParC gene length for *A. aeolicus* topo IV

Early in this project we noted that a frameshift mutation, likely due to a sequencing error, added an additional guanine nucleotide and a premature stop codon to the annotated *A. aeolicus* GyrA sequence. Our inspection of the raw sequence for this region allowed us to redefine the open reading frame to include residues 745-769 of the C-terminus. Pending acceptance of this paper and its conclusions, the *Aquifex aeolicus* GyrA gene annotation was changed to ParC in Genbank.

Tables

Table 5.1. Data collection and refinement statistics

Data Collection	Native	K ₂ Pt(CN ₄)	K ₂ PtCl ₄	NaWO ₄
Resolution (Å)	40.851-1.307	58.82-1.67	59.03-2.14	59.13-1.84
Wavelength (Å)	1.1	1.071	1.071	1.214
Space Group	P1	P1	P1	P1
Unit cell dimensions (a, b, c) (Å)	43.51, 49.62, 120.35	43.63, 49.65, 119.95	43.71, 49.51, 120.49	43.71, 49.81, 120.67
Unit cell angles (α, β, γ) (°)	91.57, 99.75, 113.48	91.48, 99.75, 113.65	91.66, 99.80, 114.08	91.99, 99.35, 113.34
I/σ (last shell)	28.2 (2.4)	12.7 (2.7)	13.7 (2.3)	10.3 (2.6)
Rmerge (last shell) (%)	3.1 (35.7)	6.2 (42.6)	4.9 (55.1)	7.0 (33.9)
Completeness (last shell) (%)	93.6 (84.8)	91.9 (68.8)	96.8 (95.1)	70.1 (69.7)
Redundancy	3.4 (2.5)	3.9 (3.2)	4.0 (3.8)	1.9 (2.0)
Unique Reflections	208116	96424	56509	55751
Number of Sites	n/a	9	7	7
Figure of Merit		0.65		
Refinement		ParC CTD		
Resolution (Å)		40.851-1.307		
No. of reflections		208073		
Rwork (%) (last shell)		17.25 (23.66)		
Rfree (%) (last shell)		18.94 (24.83)		
Structure and Stereochemistry				
No. atoms		8021		
Protein		6997		
Water		948		
Glycerol molecules		9		
Nitrate molecules		4		
B factor (Å²)				
Protein		19.98		
Water		33.51		
r.m.s.d. bond lengths (Å)		0.014		
r.m.s.d. bond angles (°)		1.501		
Ramachandran Plot (%)				
Favored Region		98.2		
Allowed Region		1.8		
Outliers		0		

$R_{\text{merge}} = \sum_{\text{hkl}} I_i(\text{hkl}) / \sum_{\text{hkl}} I_i(\text{hkl})$, where $I_i(\text{hkl})$ is the intensity of an observation and $I(\text{hkl})$ is the mean value for its unique reflection. Summations cover all reflections.

$R_{\text{work}} = \sum_{\text{hkl}} F_{\text{obs}} - k F_{\text{calc}} / \sum_{\text{hkl}} F_{\text{obs}}$. R_{free} was calculated same way as R_{work} , but with the reflections excluded from refinement. The R_{free} set was chosen using default parameters in PHENIX (28). Ramachandran Plot Categories were defined by Molprobit (30).

Table 5.2. Sequences used in MAFFT sequence alignments

Species	Gene	GI Number
<i>Mycobacterium tuberculosis</i> H37Rv	<i>GyrA</i>	1552559
<i>Tropheryma whippiei</i> str. Twist	<i>GyrA</i>	28476013
	<i>ParC</i>	28493458
<i>Streptomyces coelicolor</i> A3(Robeson, Costello et al.)	<i>GyrA</i>	8247655
	<i>ParC</i>	2661689
<i>Aquifex aeolicus</i> VF5	<i>ParC</i>	15606292
<i>Chlorobium tepidum</i> TLS	<i>GyrA</i>	21646043
<i>Thermosynechococcus elongatus</i> BP-1	<i>GyrA</i>	22295093
<i>Deinococcus radiodurans</i> R1	<i>GyrA</i>	15806912
<i>Bacillus subtilis</i>	<i>GyrA</i>	467397
	<i>ParC</i>	255767425
<i>Bacillus halodurans</i> C-125	<i>GyrA</i>	10172619
<i>Listeria monocytogenes</i> EGD-e	<i>GyrA</i>	16802055
	<i>ParC</i>	16803327
<i>Staphylococcus aureus</i>	<i>GyrA</i>	57285300
	<i>ParC</i>	57284545
<i>Thermoanaerobacter tengcongensis</i> MB4	<i>GyrA</i>	20806553
<i>Enterococcus faecalis</i> V583	<i>GyrA</i>	29342125
	<i>ParC</i>	29343636
<i>Lactobacillus plantarum</i> WCFS1	<i>GyrA</i>	28376980
	<i>ParC</i>	28378500
<i>Streptococcus pneumoniae</i> R6	<i>GyrA</i>	15458723
	<i>ParC</i>	15458351
<i>Streptococcus mutans</i> UA159	<i>GyrA</i>	24377489
	<i>ParC</i>	24377581
<i>Fusobacterium nucleatum</i>	<i>GyrA</i>	19713408
<i>Pirellula staleyi</i> DSM 6068	<i>GyrA</i>	283437591
<i>Caulobacter crescentus</i> CB15	<i>GyrA</i>	13422971
	<i>ParC</i>	2749945
<i>Agrobacterium tumefaciens</i> str. C58	<i>GyrA</i>	159184789
	<i>ParC</i>	15888496

<i>Rickettsia conorii</i> str. Malish 7	<i>GyrA</i>	15892196
	<i>ParC</i>	15892020
<i>Neisseria meningitidis</i> MC58	<i>GyrA</i>	7226624
	<i>ParC</i>	7226854
<i>Helicobacter pylori</i> J99	<i>GyrA</i>	15611708
<i>Escherichia coli</i> str. K-12 substr. MG1655	<i>GyrA</i>	16130166
	<i>ParC</i>	16130915
<i>Salmonella enterica</i> subsp. enterica serovar Typhi str. CT18	<i>GyrA</i>	16761200
	<i>ParC</i>	16761950
<i>Vibrio cholerae</i> O1 biovar El Tor str. N16961	<i>GyrA</i>	9655742
	<i>ParC</i>	9657005
<i>Thermotoga maritima</i> MSB8	<i>GyrA</i>	4981629
<i>Xylella fastidiosa</i> 9a5c	<i>GyrA</i>	9107763
	<i>ParC</i>	15837954
<i>Mycobacterium tuberculosis</i> H37Rv	<i>GyrB</i>	1552558
<i>Tropheryma whipplei</i> str. Twist	<i>GyrB</i>	28492972
	<i>ParE</i>	28493461
<i>Streptomyces coelicolor</i> A3(Robeson, Costello et al.)	<i>GyrB</i>	3036887
	<i>ParE</i>	8247656
<i>Aquifex aeolicus</i> VF5	<i>GyrB</i>	15606321
<i>Chlorobium tepidum</i> TLS	<i>GyrB</i>	21648310
<i>Thermosynechococcus elongatus</i> BP-1	<i>GyrB</i>	22298189
<i>Deinococcus radiodurans</i> R1	<i>GyrB</i>	6458625
<i>Bacillus subtilis</i>	<i>GyrB</i>	467396
	<i>ParE</i>	221309697
<i>Bacillus halodurans</i> C-125	<i>GyrB</i>	15612569
<i>Listeria monocytogenes</i> EGD-e	<i>GyrB</i>	16802054
	<i>ParE</i>	16803326
<i>Staphylococcus aureus</i>	<i>GyrB</i>	57285299
	<i>ParE</i>	57284544
<i>Thermoanaerobacter tengcongensis</i> MB4	<i>GyrB</i>	20514990
<i>Enterococcus faecalis</i> V583	<i>GyrB</i>	29342124

	<i>ParE</i>	29343637
Lactobacillus plantarum WCFS1	<i>GyrB</i>	28376979
	<i>ParE</i>	28378501
Streptococcus pneumoniae R6	<i>GyrB</i>	15902759
	<i>ParE</i>	15458350
Streptococcus mutans UA159	<i>GyrB</i>	24377654
	<i>ParE</i>	24379635
Fusobacterium nucleatum	<i>GyrB</i>	19705416
Pirellula staleyi DSM 6068	<i>GyrB</i>	283778833
Caulobacter crescentus CB15	<i>GyrB</i>	13421273
	<i>ParE</i>	2749947
Agrobacterium tumefaciens str. C58	<i>GyrB</i>	159184123
	<i>ParE</i>	159184842
Rickettsia conorii str. Malish 7	<i>GyrB</i>	15892807
	<i>ParE</i>	15892232
Neisseria meningitidis MC58	<i>GyrB</i>	7225433
	<i>ParE</i>	7226935
Helicobacter pylori J99	<i>GyrB</i>	15611520
Escherichia coli str. K-12 substr. MG1655	<i>GyrB</i>	49176395
	<i>ParE</i>	16130926
Salmonella enterica subsp. enterica serovar Typhi str. CT18	<i>GyrB</i>	16762490
	<i>ParE</i>	16761956
Vibrio cholerae O1 biovar El Tor str. N16961	<i>GyrB</i>	9654406
	<i>ParE</i>	9657006
Thermotoga maritima MSB8	<i>GyrB</i>	4981365
Xylella fastidiosa 9a5c	<i>GyrB</i>	9104765
	<i>ParE</i>	9106275
Chlamydophila pneumoniae CWL029	<i>GyrB</i>	4376548
	<i>ParE</i>	15618625
Chlamydia trachomatis 434/Bu	<i>GyrB</i>	157060424
	<i>ParE</i>	165929991

Table S2. GyrA, ParC, GyrB, and ParE sequences used in Figures S4 and S5. Genes are listed as annotated in the NCBI database (www.ncbi.nlm.nih.gov). The 4 *Chlamydia* genes were omitted from the GyrA/ParC alignments because they lack a C-terminal domain (CTD).

Figures

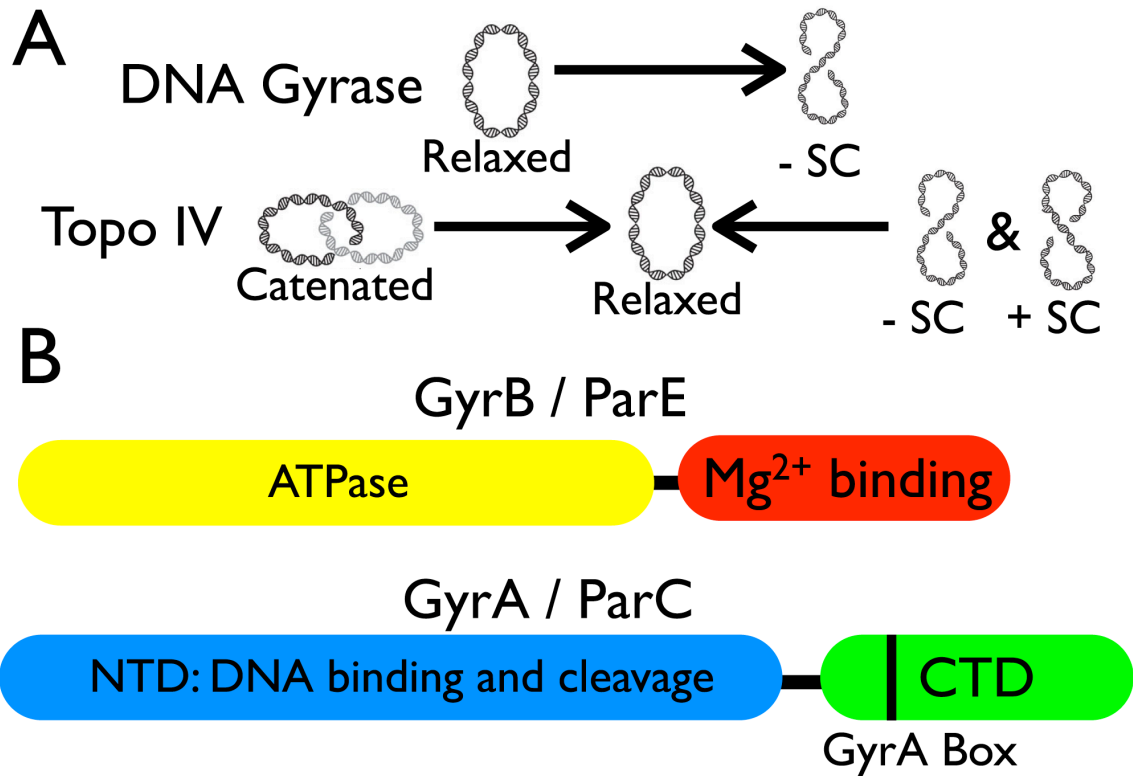


Figure 5.1. Type IIA topoisomerase functions and structure. Hallmark type IIA topoisomerase activities (A) and primary domain structures (B) of bacterial type IIA topoisomerases. Domains are colored and domain functions are indicated with labels.

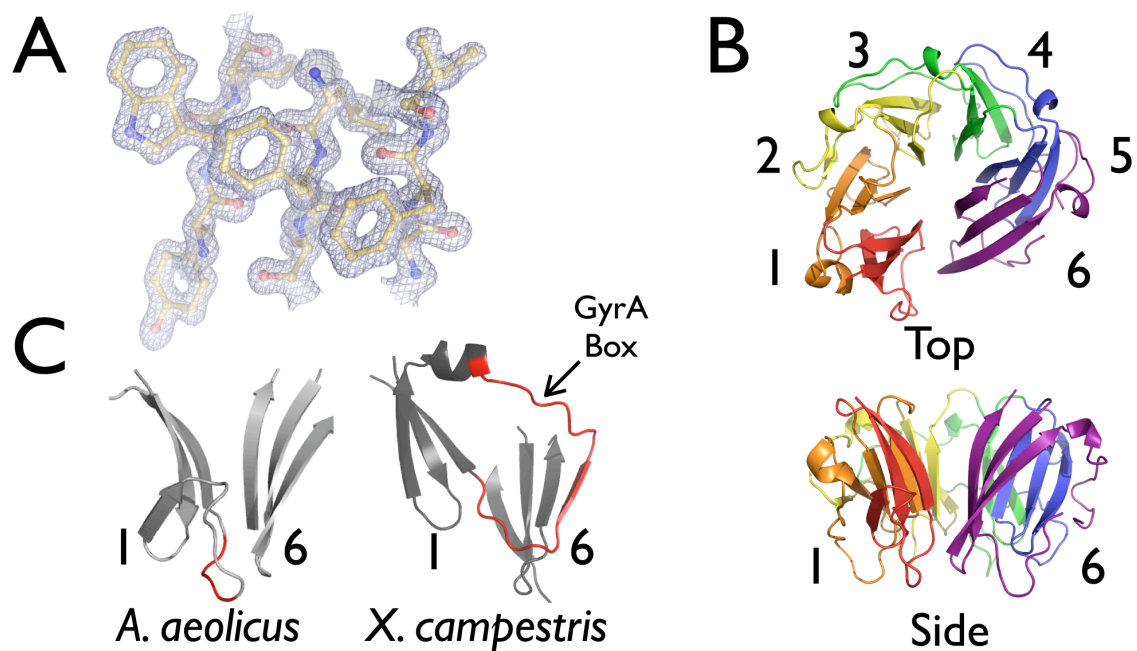


Figure 5.2. *A. aeolicus* CTD structure. (A) Representative electron density from a refined 2Fo-Fc map contoured at 1.8σ . (B) Ribbon model of *A. aeolicus* CTD. Blades are numbered. (C) Comparison of blades 1 and 6 of the *A. aeolicus* and *X. campestris* CTDs highlights the missing *A. aeolicus* GyrA-box, shown in red in both structures.

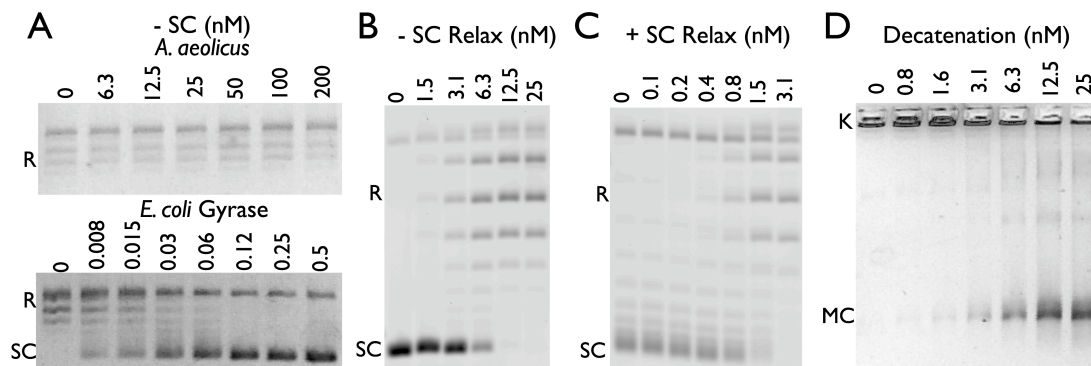


Figure 5.3. *A. aeolicus* holoenzyme assays. (A) Negative supercoiling activity. *E. coli* gyrase activity is shown for comparison. Negative (B) and positive (C) supercoil relaxation. (D) Decatenation. DNA species are labeled as follows: SC – supercoiled, R – relaxed, K – intact kDNA network, MC – released kDNA minicircles.

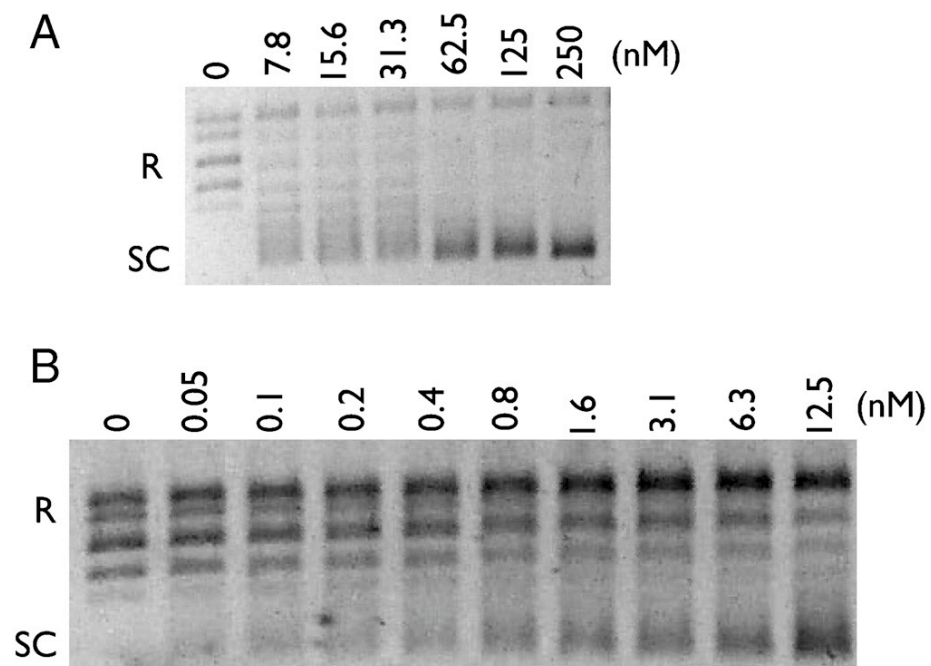


Figure 5.4. Negative supercoiling chimera activities. (A) Subunit mixing—*E. coli* GyrA with *A. aeolicus* GyrB. (B) *A. aeolicus* NTD fused to the *T. maritima* CTD.

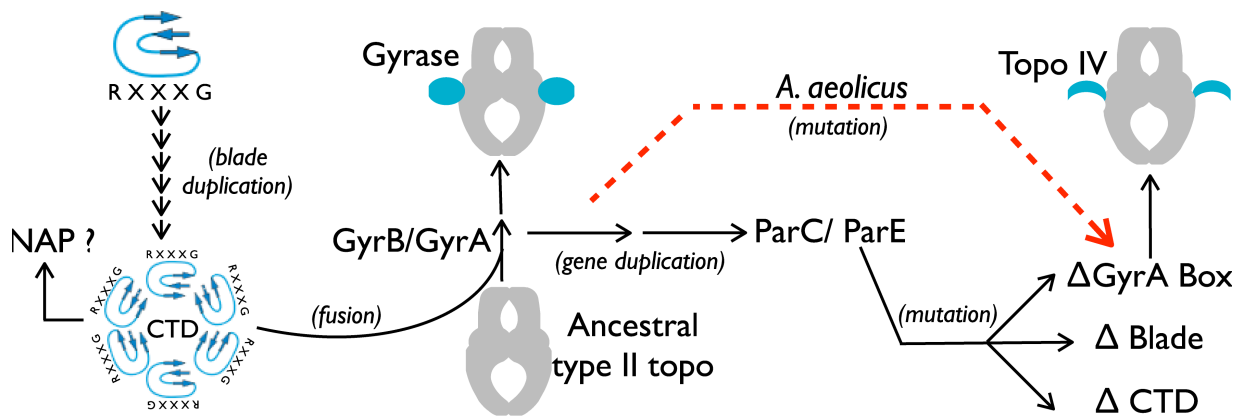


Figure 5.5. Model for bacterial type IIA DNA topoisomerase evolution and specialization.

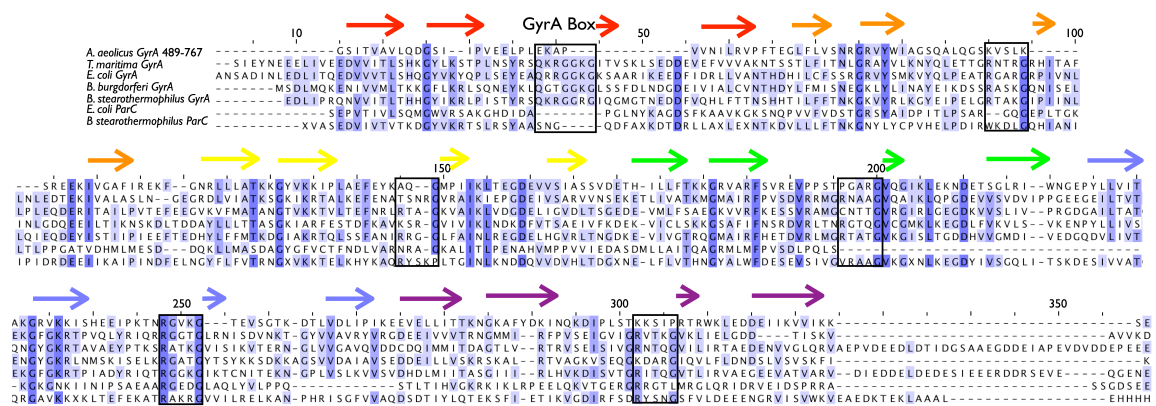


Figure S5.1. Sequence alignment between representative CTDs. Secondary structure elements are shown with arrows above and are colored by blade. The GyrA Box in the first blade and the degenerate GyrA Box motifs in subsequent blades are boxed in black.

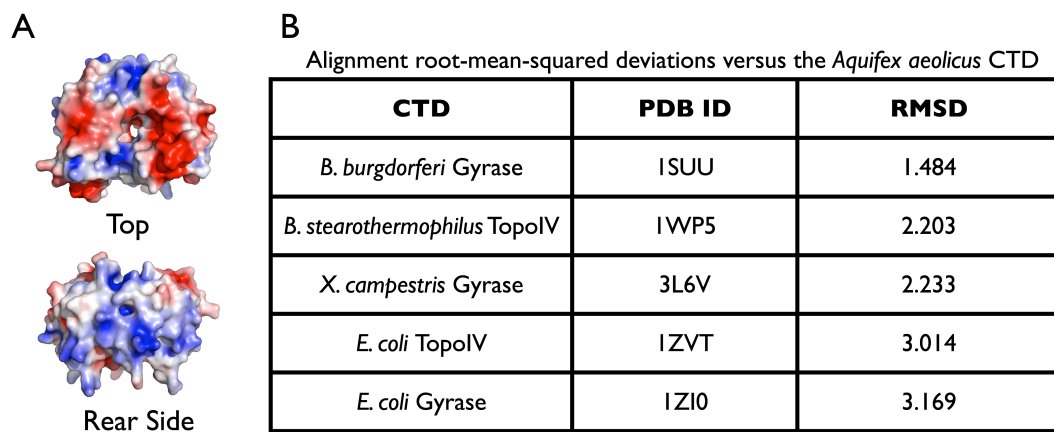


Figure. S5.2. C-terminal domain surface charges and alignment statistics. (A) Electrostatic surface representation of the *A. aeolicus* CTD. (B) Table of root-mean-squared deviations for alignments between available type IIA topoisomerase CTD structures and the *Aquifex aeolicus* CTD. Electrostatic surface representation and alignments were performed using Pymol (48).

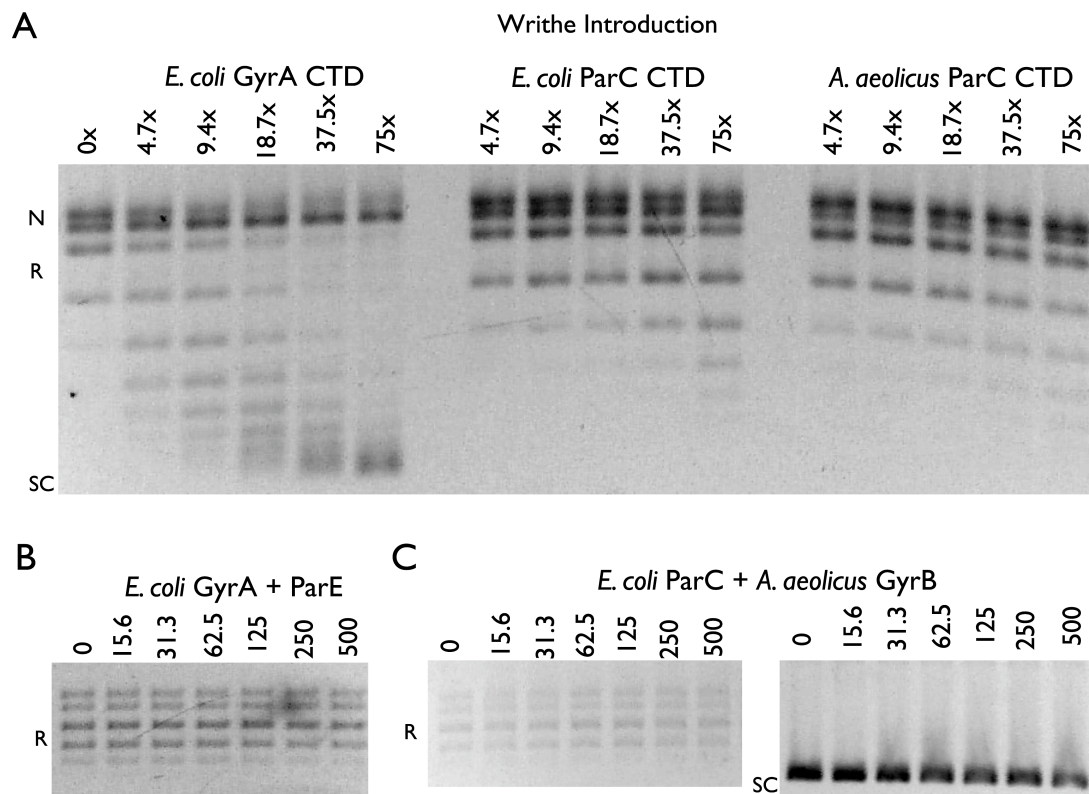


Figure S5.3. Topoisomerase DNA wrapping and activity assays (A)

Topology footprint assays. Relaxed pUC19 (6 nM) was incubated with varying amount of CTD (indicated as a molar ratio of CTD:pUC19). Topoisomer labels: N – Nicked, R – relaxed, SC – supercoiled. (B) Chimeric activity assays. *E. coli* ParC/*A. aeolicus* GyrB negative supercoiling assay (left) and negatively-supercoiled DNA relaxation (middle). *E. coli* GyrA/*E. coli* ParE negative supercoiling assay (Chakraverty and Wright).

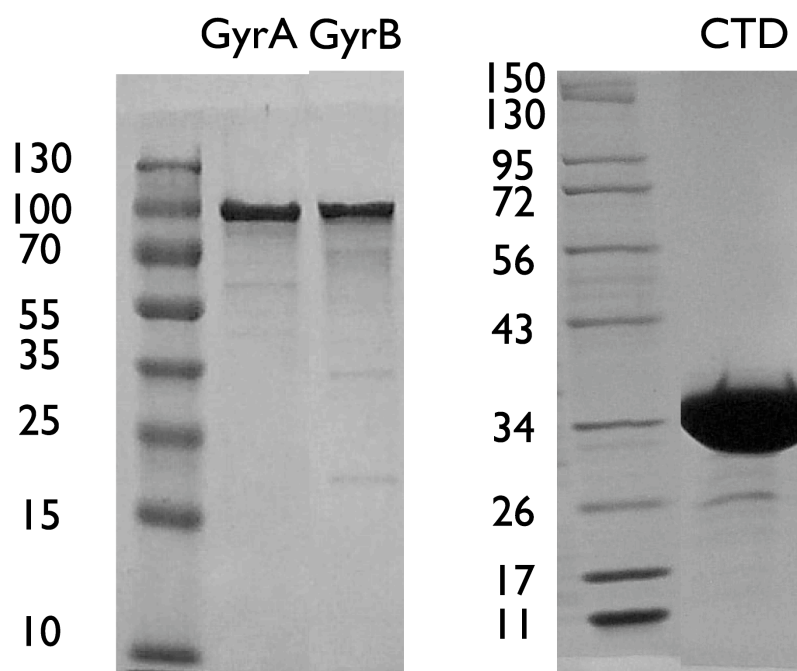


Fig. S5.6. SDS-PAGE gels of purified *A. aeolicus* proteins.

Chapter 6 - Conclusions and Future Directions

(Portions reproduced from: Tretter, E.M. and Berger, J.M. (2012). J Biol Chem (In Press), Tretter, E.M. and Berger, J.M. (2012). J Biol Chem (In Press) Tretter, E.M., Lerman, J.C., and Berger, J.M. (2009). Proc Natl Acad Sci 107, 22055-22059)

Conclusions

Key cellular processes, such as DNA replication and transcription, result in the supercoiling of genomic DNA, which is resolved by molecular machines known as topoisomerases (**Chapter 1**). Despite extensive study, the mechanisms used to modulate and tune bacterial type IIA topoisomerase function are still poorly understood. In this work I introduce evidence that multiple adaptations to the CTD refine enzyme activity (**Chapter 2, 4**), characterize novel regulatory mechanisms (**Chapter 3**), and identify novel type IIA topoisomerase evolutionary paths (**Chapter 5**).

The majority of bacteria code for two heterotetrameric (A_2B_2) type IIA topoisomerases (Huang 1996; Forterre, Gribaldo et al. 2007; Schoeffler and Berger 2008). The underlying basis for this duplication may derive from a need to divide the labor of DNA supercoiling and chromosome decatenation between distinct enzymes. The topoisomerase required for supercoiling, DNA gyrase, is more widespread than its counterpart used in chromosome segregation, topo IV. Moreover, in those species that contain a single type II topoisomerase, such as *Mycobacterium tuberculosis*, this enzyme up until now always has been shown to be a *bona fide* gyrase (**Chapter 5**) (Manjunatha, Dalal et al. 2002). There had been significant debate as to whether topo IV preceded gyrase or whether the enzyme is a degenerate form of gyrase (Corbett, Schoeffler et al. 2005; Forterre, Gribaldo et al. 2007). A key point of distinction between these two paralogs, aside from general sequence divergence, derives from their C-terminal domains (CTD), which in some topo IV orthologs is missing entirely (Corbett, Schoeffler et al. 2005).

In Chapter 3 I identified a novel regulatory element, an unconserved C-terminal tail, in *E. coli* GyrA that controls DNA binding, wrapping, and supercoiling setpoint. Since the GyrA tail is not conserved (**Figure 3.1**), it is unlikely that the mechanism by which it controls *E. coli* gyrase will be universal. Indeed, differences in supercoiling between gyrase orthologs have been observed previously (Blanche, Cameron et al. 1996; Aubry, Fisher et al. 2006; Champion and Higgins 2007) (**Chapter 4**), and have been attributed to disparate factors such as alterations in GyrB sequence or CTD structure. These studies indicate that gyrase is not a monolithic entity, and that instead there exists a range of gyrase activities that vary in their relative supercoiling efficiencies. They also suggest that rather than being the rule in terms of supercoiling efficiency, *E. coli* gyrase is a supercharged exception.

In Chapter 4 I determined that the supercoiling setpoints of *E. coli* and *M. tuberculosis* gyrase differ due to *M. tuberculosis*' diminished ATPase activity and a lack of coupling between ATP binding and DNA wrapping. This concept is in line with recent findings in other bacterial systems; for example, *E. coli* and *S. typhimurium* gyrase

appear to have evolved different supercoiling setpoints as a means to accommodate different rates of transcription (Booker, Deng et al. 2010), while the “gyrase” of *Aquifex aeolicus* appears to have undergone a relatively recent conversion into a topo IV, possibly to accommodate life at hyper-thermophilic temperatures (**Chapter 5**) (Forterre, Bergerat et al. 1996; Tretter, Lerman et al. 2010). The slow rate of turnover exhibited by *Mtb* gyrase may also play a role in controlling the likelihood of sampling *cis* and *trans* strand passage events: a long dwell time in an inactive state where DNA wrapping and ATPase status are uncoupled would favor unwrapping, and thus loss of the transported segment in *cis*, disfavoring supercoiling as a nucleic acid substrate becomes progressively more underwound. In addition, there is growing evidence of specific functional and architectural differences between type IIA topoisomerases orthologs. A unique domain insertion found in the GyrB subunit of γ -prokaryotes that impacts DNA binding serves as one example (Schoeffler, May et al. 2010), whereas an acidic tail appended the C-terminus of *E. coli* GyrA that controls DNA wrapping is another (**Chapter 4**).

Future Directions

While this work provides novel insight into bacterial type IIA topoisomerase specialization, it also paves the way for new research. Despite extensive study, it is often unclear from phylogenetic information alone which modifications impact gyrase function and how. Further analysis of gyrase orthologs from a range of bacterial species with distinct physiologies and environmental niches will be needed to more fully grasp the myriad of means by which evolution appears to have specialized type IIA topoisomerase function. For example, characterization of other deeply-rooted organisms that are optimized for life's extremes may provide further insights to the path topoisomerases traveled to become the highly-specialized enzymes that exist today. Characterization of gyrase orthologs using single molecule techniques could provide detailed information on how modifications to gyrase impacts enzyme timing, coupling, and rate.

It is presently unclear how the *E. coli* GyrA tail might selectively bind to different regions of gyrase to control holoenzyme conformation and function. Additionally, the C-terminal domain tail is highly variable across type IIA topoisomerases and it is unclear if this domain is modified to tune topoisomerase function in other bacterial species. It also is not understood how or why particular bacterial species adjust and select their respective topoisomerase retinue to achieve a particular level of steady-state supercoiling. Issues such as these are subjects for future investigations.

To further understand the biochemical basis for bacterial type IIA topoisomerase paralog specialization I have prepared *E. coli* gyrase/topo IV chimeras where the CTD and its accompanying linker from each enzyme have been replaced with the accompanying paralog's CTD and linker. Characterization of these chimeras may shed additional light to the modifications required for intraspecies topoisomerase specialization. Determining the rules for both bacterial type IIA paralog and ortholog specialization will provide new clues on how these molecular machines evolved and will assist the search for novel antibiotics.

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Appendix

Auto-Induction Protein Expression

(Adapted from: Studier, F.W. (2005) Protein Expr Purif. 41, 207-234.)

Transformation:

- Transform into B121 (dE3) or BL21 (DE3) RIL cells
- Pick single colonies into non-inducing media
- Shake 37°C degrees overnight

Expression:

- Prepare expression culture of auto-inducing media
 - Use double amp/kan resistance (1x clr)
 - Use your usual flasks/volumes
- Inoculate expression culture of auto-inducing media
- Grow culture 3-4 hours, shaking at 37°C degrees (OD 0.4-0.6)
- Move culture to room temperature and grow for another 12-24 hours
 - Check OD, expression, solubility at 12 & 24 hours
- Re-suspend cells in lysis buffer
- Drop freeze or Sonicate

Non-inducing media (MDG)– Autoclave water, then add:

<u>200 ml</u>	<u>1L</u>	
4 ml	20 ml	50xM yielding: 25mM Na ₂ HPO ₄ 25mM KH ₂ PO ₄ 50mM NH ₄ Cl 5mM Na ₂ SO ₄
0.2 ml	1 ml	2M MgSO ₄ yielding: 2mM MgSO ₄
40 ul	0.2 ml	1000x trace metals yielding: 0.2x trace metals
2.5 ml	12.5 ml	40% w/v glucose yielding: 0.5% w/v glucose
10 ml	50 ml	5% w/v neutralized aspartic acid yielding: 0.25% w/v aspartate

Auto-inducing media (ZYM-5052):

1L

Autoclave:

10 g tryptone yielding 1% w/v N-Z amine or tryptone

5 g yeast extract yielding 0.5% w/v yeast extract

Add after autoclave:

20 ml 50xM yielding:

	25mM Na ₂ HPO ₄
	25mM KH ₂ PO ₄
	50mM NH ₄ Cl
	5mM Na ₂ SO ₄
1 ml	2M MgSO ₄ yielding: 2mM MgSO ₄
0.2 ml	1000x trace metals yielding: 0.2x trace metals
20 ml	50x5052 yielding: 0.5% w/v glycerol 0.05% w/v glucose 0.2% w/v α-lactose

Trace Metal Component Stocks:

Sterile Filter:

400mM CaCl₂-2H₂O

2.94g in 50 ml

200mM MnCl₂-4H₂O

1.98g in 50 ml

200mM ZnSO₄-7H₂O

2.88g in 50 ml

200mM CoCl₂-6H₂O

2.38g in 50 ml

200mM CuCl₂-2H₂O

1.7g in 50 ml

200mM NiCl₂-6H₂O

2.38g in 50 ml

200mM Na₂MoO₄-2H₂O

2.42g in 50 ml

200mM Na₂SeO₄

1.73g in 50 ml

200 mM H₃BO₄

0.62g in 50 ml

1000x trace metals (100ml yields 500 liters of culture):

- Make 100-fold dilution of concentrated HCl (~0.12M HCl)

- Dissolve FeCl₃ in this to 0.1M

- To 50 ml of the FeCl₃ solution add 1 ml of 0.2M stocks of CoCl₂, CuCl₂, NiCl₂, Na₂MoO₄, Na₂SeO₄, H₃BO₄

- Add 5 ml of 0.2M stocks of MnCl₂, ZnSO₄

- Add 5ml of 0.4M stock of CaCl₂

- Add water to 100 ml

Sterile Filter

Final Stock Concentrations:

20mM CaCl₂-2H₂O/10mM MnCl₂-4H₂O/10mM ZnSO₄-7H₂O/2mM CoCl₂-6H₂O/2mM

CuCl₂·2H₂O/2mM NiCl₂·6H₂O/2mM Na₂MoO₄·2H₂O/2mM Na₂SeO₄/2 mM H₃BO₄

50x5052 (autoclave) (1 Liter yields 50 Liters of culture):

25% w/v glycerol (250 ml in 1 liter)

2.5% w/v glucose (25g in 1 liter)

10% w/v α-lactose monohydrate (100g in 1 liter)

50xM (autoclave) (1 Liter yields 50 Liters of culture):

1.25M Na₂HPO₄ 335.1g heptahydrate in 1 liter

1.25M KH₂PO₄ 170.1g anhydrous in 1 liter

2.5M NH₄Cl 133.7g anhydrous in 1 liter

0.25M Na₂SO₄ 35.5g anhydrous in 1 liter

2M MgSO₄

49.3 heptahydrate in 100ml (autoclave or sterile filter) 100 ml yields 100 liters of culture

40% w/v glucose

100g in 250 ml (autoclave) 80x: 250 ml yields 20 liters of culture

5% w/v NaOH neutralized aspartic acid: 12.5g in 250ml, neutralize with NaOH
(autoclave) 20x: 250 ml yields 5 liters of culture

Large-scale *in-vivo* recombination to generate minicircle DNA

Cell and Plasmid Stocks:

LZ54 Cells- with (pmc339-BbvCI (Ampicillin)) and without plasmid- for transforming with your own plasmid minicircle.

pMC339- plasmid makes 339bp minicircle w/ BbvCI nick site

pMC-MCS- plasmid for generating minicircle containing multiple cloning site

DH5 α -pMC-MCS- minicircle in DH5 α for making more parent plasmid

DH5 α -pMC339- minicircle in DH5 α for making more parent plasmid

Transforming into LZ54 using the CaCl₂ method:

(LZ54 has integrase under the temperature sensitive lambda cI repressor, which falls off at 37°C and ultimately results in cell death)

- Grow and heat shock cells at 30°C (NOT 42°C)

- Heat shock cells for 5 min at 30°C then ice for a 2 minutes and return to 30°C for 1 hour

-Transform miniprep DNA into LZ54 to increase transformation efficiency

Starter Cultures:

2x 1 L LB

100 ml LB

Fermentor Media- Make in 2x 2 L bottles

48 g tryptone

192 g yeast extract

120 ml glycerol

0.4 ml antifoam 204 (Sigma) (add after autoclave)

3.48 L H₂O

1 L LB: 10g Tryptone, 5g Yeast Extract, 10g NaCl

400mL Potassium Phosphate Solution- (add to media in fermentor)

9.28 g potassium phosphate monobasic (KH₂PO₄)

50.16 g potassium phosphate dibasic (K₂HPO₄)

Day 1:

Make LB media and Fermentor Media

20 ml LB + 100 ug/ml ampicillin overnight culture w/ LZ54-pMC

Grow o/n @ 30° standing

Day 2:

Use Day 1 o/n to inoculate 2x 1L LB + 100 ug/ml ampicillin (in 1L shaking flasks).

Grow o/n @ 30° shaking

Day 3:

Add Fermentor Media and Potassium Phosphate Solution to fermentor

Set to 30°

Set dO₂ to 30%, control by agitation (low at 500 rpm, high at 1000 rpm)

Harvest 1L cells by centrifugation in 1 L bottles (2)

Resuspend pellets in a total of 50 ml fresh LB

Add resuspended cells to fermentor.
 Check A600 regularly, lag phase should be minimal
 During log phase, LZ54 doubles approx once every hour @ 30°
 Grow cells to mid-log
 This is OD 3.0 for the MacroLab Fermentor
 Take 1ml of culture (mid-log sample)
 Increase temperature to 42°
 Maintain this temperature (once reached) for 30 minutes
 Take 1ml cell sample (Integrase expressed but inactive)
 Set temperature setting back to 30°
 Once reached, keep @ 30° for 60 minutes
 Take 1ml cell sample (Integrase active)
 Harvest cells by centrifugation
 Check recombination before starting large-scale prep by mini-prepping 1 ml
 cell samples
 Cleave 10ul of miniprep DNA w/ BamHI and resolve on 1% agarose gel
 Use ratio of bands to determine fraction recombined

pMC-MCS Multiple Cloning Site

Minicircle sequence highlighted in bold (and colored red and blue).

Sequencing Primer Sequences

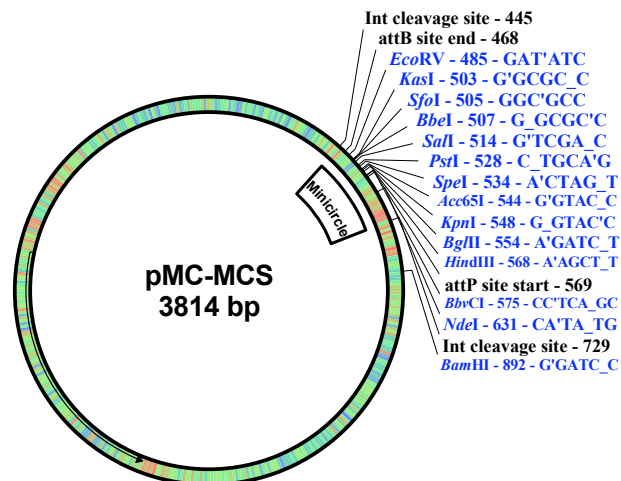
Sites for I-integrase recombination in red (do not alter this region, required for generating minicircle).

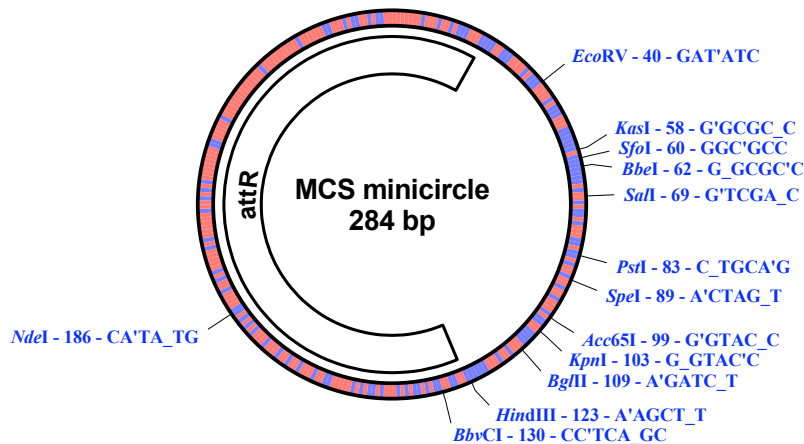
Sequence of interest may be cloned into region highlighted in blue (includes multiple cloning site).

```
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Plasmid map





Forward Seq Primer:

ACCAGTGACGAAGGCTTGAG order: ACCAGTGACGAAGGCTTGAG 284bp upstream of int site

Reverse Seq Primer:

TATCCGCTCACAATTCCACA order: TGTGGAATTGTGAGCGGATA 271bp downstream of int site

Full length (parent) plasmid 339bp- with BbvCI nicking site

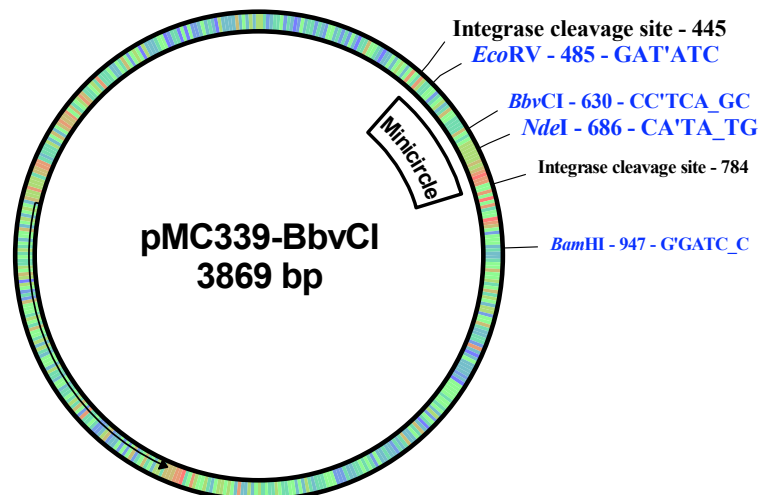
Minicircle sequence highlighted in bold (and colored red and blue).

Sites for I-integrase recombination in red (do not alter this region, required for generating minicircle). Sequence of interest may be cloned into region highlighted in blue

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GGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCG

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 CGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCT
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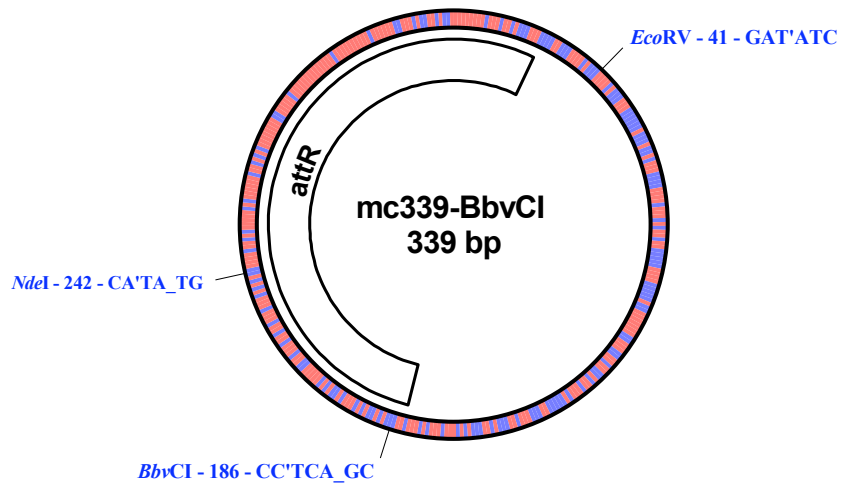
Plasmid map



Minicircle sequence

TTTATACTAACTTGAGCGAAACGGAAGGGTTTTTCACCGATATCACCGAAACGCGGAGGCAGCTGTATGG
 CGAAATGAAAGAGTTCTTCCCGGAAACGCGGTGGAATATTTTCGTTTCTACTACGACTACTATCAGCCGG
 AAGCCTATGTACCGAGTTCCGACACTTTCATTGAGAAAGATGCCTCAGCTCTGTTACAGGTCACTAATACC

ATCTAAGTAGTTGATTCATAGTGACTGCATATGTTGTGTTTTACAGTATTATGTAGTCTGTTTTTTATGCA
AAATCTAATTTAATATATTGATATTTATATCATTTCGTTTCAGCTTT



DNA Minicircle Purification

1: Alkaline Lysis

-Resuspend cells in ~20mL **GLEDT buffer**. Add to 480mL **GLEDT buffer** and add 0.5g lysozyme. Incubate at room temperature for 30 minutes, stirring slowly.

-Lyse cells by adding 1L **Lysis Buffer**. Mix slowly and incubate at room temperature for 5 minutes. *Do not allow lysis to proceed longer than 5 minutes to avoid permanent DNA denaturation.

-Precipitate proteins etc. by adding 750ml **Protein Ppt Buffer**. Mix slowly. *Lysate may be stored at 4°C overnight.

-Pellet the precipitate by centrifugation for 30min at 7k using the GS3 rotor.

-Filter supernatant through gauze in Buchner funnel. Aliquot supernatant to 500ml centrifuge bottles and add **isopropanol** (>0.7x vol.) (250ml sup/180ml isopropanol) to precipitate nucleic acid (Use 6 bottles and reuse 2 after first spin). Invert tubes to mix and incubate 10 minutes at room temp. Spin for 30 min at 7k.

-Pour out supernatant, remove all with pipet and let pellets dry for 10+ minutes upside down on paper towels.

2: LiCl treatment (Removes high molecular weight RNA)

-Resuspend nucleic acid in a total volume of 120mL **TE pH8**.

*Shake tubes @37°C to aid resuspension.

-Add an equal volume of **5M LiCl** to precipitate high molecular weight RNA. Spin bottle for 15 minutes at 7k and transfer supernatant to 2 clean 500mL centrifuge bottles.

Discard RNA pellet. Add 2x volume ethanol to precipitate nucleic acid (do not add additional salt). Incubate at -20°C for 30 minutes or longer.

*Nucleic acid may be stored at -20°C overnight or longer.

-Spin bottles for 30 minutes at 8k to pellet nucleic acid. Remove supernatant and air dry pellets for 10 minutes.

-Resuspend nucleic acid in 150mL **no salt buffer** and add RNaseA to 50 ug/ml (7.5mg) to degrade low molecular weight RNA. Incubate at 37°C for 30 minutes.

-Add Proteinase K to 50 ug/ml (7.5mg) and incubate for 30 minutes.

*Sample may be stored at 4°C overnight. **Sample 1, 30ul

3: PEG precipitation of large plasmids

*PEG precipitation should be followed immediately by column purification.

- Add 150mL **PEG Buffer**. Incubate at 4°C for 10 minutes. Spin at 8k for 20 minutes. Transfer supernatant to a new bottle.

**Sample 2, 60ul

-Add 20 mL of **no salt buffer** to adjust [NaCl] to 750mM. Add 180mL anion exchange **Load Buffer** to reduce PEG concentration.

**Sample 3, 100ul

- Proceed immediately to the anion-exchange purification. It is not advisable to leave the DNA in the PEG supernatant for a prolonged period of time as the minicircle may begin to precipitate.

4: Qiagen Gigaprep anion-exchange column

*Order columns only “Qiagen Tip 10,000”

*Recipes for buffers are in the Qiagen Gigaprep protocol book.

*Add 5mM EDTA (final conc) to all buffers to minimize DNA nicking.

-During PEG precipitation step equilibrate a Qiagen-tip 10,000 column with 100ml of buffer **QBT**.

-Apply supernatant from PEG precipitation step. Collect flow-through.**Sample 4, 100ul

-Wash with 600ml buffer **QC**. **Sample 5, 120ul

-Elute with 120ml buffer **QN**. Collect eluate in clean bottle. ** Sample 6, 20ul

*Eluate may be stored at 4°C overnight.

-Reduce volume of eluate to <1ml using centrifuge concentrators (Millipore Amicon Ultra, 30,000MW cutoff, but 50,000 is also fine). Buffer exchange into **TE** while concentrating DNA. Freeze or proceed to S-500 gel filtration column.

Purification Analysis (optional)

-DNA yield and quality may be easily analyzed by agarose gel electrophoresis. Take samples 1-6 and adjust volume to 200ul. Extract with phenol/chloroform to remove PEG (PEG will otherwise distort DNA mobility). Extract with chloroform and EtOH precipitate. Pellet DNA, wash with 70% EtOH and dry. Resuspend samples in 20ul TE and load 10ul on a 1% agarose gel.

5: Gel filtration, Sephacryl S-500 column

-Equilibrate room temperature S-500 column in **S-500 buffer**.

-DNA should be in a total volume of <1ml TE. Spin in microcentrifuge at full speed for 2 minutes to remove particulate and transfer to a new tube. Use a syringe to load DNA into a 2ml loop on the room temperature FPLC.

*S500 Column can be equilibrated at 2ml/min & run with sample at 1ml/min.

-Collect 4ml fractions for 150ml. Run 15ul of each fraction containing nucleic acid (A280 reading will be low but informative) on a 1% agarose gel to identify the fractions you will pool and concentrate.

*Gel filtration may be repeated to reduce amount of dimer minicircle.

* Concentrate fraction to <1ml using centrifuge concentrators, further concentration can be achieved via SpeedVac.

DNA topoisomers: Mostly -2/~5% -1/~3% -3

6: Preparative gel electrophoresis

* This step is only necessary when a pure, homogenous preparation of a single topoisomer is required. Preparative gel electrophoresis typically leads to a loss of yield (up to 50%).

-EtOH ppt DNA and resuspend in 300ul TE. Check yield by absorbance.

- Pour 5% native polyacrylamid gel, 29:1 (acrylamide: bis-acrylamide), 3mm thickness, 16x20 cm. Gel buffer: Tris-borate (90mM Tris base, 90mM boric acid) + 10mM CaCl₂

-Load DNA on gel across all lanes (Add 100ul glycerol gel loading buffer to DNA before loading gel). Run for 1000Vhr at <140V (can run overnight). Buffer should be continuously recirculated. Visualize DNA by UV shadowing and excise bands.

-Elute DNA by crush and soak: Crush DNA slices by forcing it through a syringe into a 50mL falcon tube. Add 25mL TE buffer and gently agitate overnight at room temperature. Remove supernatant and transfer to a new tube. Add 10mL TE to crushed pieces and agitate for several hours to recover remaining DNA. Remove supernatant, filter into new tube and combine with original natant from first soak. Concentrate by centrifugal filtration. Extract with phenol chloroform and then chloroform. EtOH precipitate. Resuspend in TE buffer (approx 500ul)

Purification Buffers:

GLEDT		
500 ml	4.5 g	50 mM Glucose
	10 ml	10 mM EDTA
	12.5 ml	25 mM Tris pH 8
Lysis Buffer		
1L	10 g	1% SDS
	8 g	0.2 M NaOH
Protein Ppt		
750 ml	228.84 g	3M Potassium Acetate
	86.25 ml	11.5% Glacial Acet. Acid
Isopropanol		~1.4L total
No Salt		
170 ml	8.5 ml	50 mM MOPS pH 7
	1.7 ml	5 mM EDTA
LiCl₂		
120 ml		5M LiCl ₂ Final
PEG Buffer		

150 ml	15 g	10% PEG- 8000
	60 ml	1.6M NaCl
Load Buffer		
180 ml	9 ml	50 mM MOPS pH 7
	33.75 ml	750 mM NaCl
	1.8 ml	5 mM EDTA
QBT		
100 ml	18.75 ml	750 mM NaCl
	5 ml	50 mM MOPS pH 7
	15 ml	15% Isopropanol
	150 μ l	0.15% Triton X-100
	1ml	5 mM EDTA
QC		
600 ml	150 ml	1 M NaCl
	30 ml	50 mM MOPS pH 7
	90 ml	15% Isopropanol
	6 ml	5 mM EDTA
QN		
120 ml	48 ml	1.6M NaCl
	6 ml	50 mM MOPS pH 7
	18 ml	15% Isopropanol
	1.2 ml	5 mM EDTA
S-500		
500 ml	5 ml	10 mM Tris 8.0
	1 ml	1 mM EDTA
	12.5 ml	100 mM NaCl
TE		
200 ml	2 ml	10 mM Tris, pH 8.0
	400 μ l	1 mM EDTA, pH 8.0

kDNA Purification

(Adapted from: Shapiro, T.A., Klein, V.A., Englund, P.T. Methods Mol Biol. (1999) 94, 61-67.)

C. fasciculata cells can be purchased from the ATCC.

Thaw Stock Culture:

- Thaw tube in 35°C bath with cells above the waterline. (2-3) minutes
- Aseptically transfer cells to 2 100ml cultures (divide in half) in 500+ml flasks

100ml Culture Growth:

- 4+ days, Moderate speed, 25°C

Cryopreserve Culture: [this didn't work- cells did not grow when re-thawed]

- at 24hrs of growth
- Dilute midlog-phase culture with equal volume freezing media
- Dispense aliquots into cryo tubes
- Freeze incrementally: Ice 20 min, -20°C 1 hr, flash freeze in liquid Nitrogen, store at -80°C

Harvest Cells: (Media will be cloudy, and by four days this will stop increasing)

- I never quantitated cell counts before harvesting.
- 1ml culture into 100ml fresh media (if you wish to inoculate more cultures)
- SS34 15k 10 minutes at 4°C
- Wash Pellet with NET-100, Resuspend ~1g aliquots of cells and store at -80°C

Lyse Cells:

- Resuspend 2g cells in 20ml lysis buffer
 - 0.1 mg/ml prot K (2mg), 1% SDS [all in Net-100]
- Incubate in 50°C bath in 50ml falcon tube 2 hours, shake gently periodically
- shear lysate by pressing through an 18 gauge needle
- layer lysate on 4 20% sucrose (in Net-100) tubes SW-28 polyallomer tubes
- Centrifuge 20k RPM 20°C 30 minutes – do NOT select rotor on centrifuge
- Carefully remove sucrose (soft and mobile pellet)
- Pool pellets in TE (~10ml) re-layer 2 SW-28 polyallomer tubes on 20% sucrose (in Net-100), same time & speed
- Remove sucrose gradient, carefully pool pellets in ~1ml TE
- dialyze 4°C o/n in 1L TE.

Test Purity with 1% Agarose Gel:

- kDNA will stay in well but contaminating nuclear DNA will be visible as a smear - XhoI digest (cuts most minicircles at 2.5kb)

-A third sucrose gradient can be used to remove residual genomic DNA

NET-100: 250mL

- 1.45g NaCl (100mM)

- 50ml of 500mM EDTA pH 8 (100mM)
- 2.5ml Tris HCl pH 8
- add water to ~150ml, adjust pH to 8.0 bring volume to 250mL

TE: 1L

- 10ml of 1M Tris pH 8.0 (10mM Tris pH 8.0)
- 2ml of 500mM EDTA (1mM EDTA pH 8.0)

Growth Media:

- 37g/L Autoclaved BHI (Brain Heart Infusion)
- with: Hemin stock added 1:100 v:v to sterile BHI media.

Hemin Stock:

- 2mg/ml in 50mM NaOH, filter sterilize (use within ~2 weeks)

Freezing Media: 10mls

- 1.5ml glycerol, .37g BHI

Chloroquine Gels

To resolve negatively supercoiled topoisomers on crisp agarose gels:

- Start with 3 $\mu\text{g}/\text{ml}$ chloroquine in agarose gel and running buffer
 - 10 mg/ml stock stored at 4°C in the dark
- Gel: 1% agarose made with fresh 50x TAE stock
 - Incubate gel after microwaving in $\sim 50^{\circ}\text{C}$ bath for 10-15+ min
 - Pre-incubate chloroquine at $\sim 50^{\circ}\text{C}$ bath for ~ 5 min before adding to gel
- Run gel ~ 44 Volts 18-20 hours on large gel box w/ buffer pump (start pump ~ 40 minutes after gel starts)
- Protect chloroquine gel from light with foil.
- Large gel box: 200 mL 1% agarose gel in 1x TAE & 1.5 L 1x running buffer
- Wash gel in TAE 20 min (remove excess chloroquine)
- Stain with .5 $\mu\text{g}/\text{ml}$ Ethidium Bromide in H_2O 30 min (5 $\mu\text{l}/100$ ml)
- Destain in H_2O 25-40 min

Gel Sample Preparation:

- If you have KCl in your assay buffer (or any salt that is insoluble in SDS)
 - Stop reaction with your stop buffer, add loading dye and place on ice for ~ 10 min.
 - Spin reaction in bench top centrifuge at top speed to pellet precipitate.
 - Load samples onto chloroquine gel, avoiding the precipitate pellet.

Topoisomerase Writhe Assay

Topo IB Protocol:

- 10 minute protein/DNA incubation 37°C (mix w/ p20 15ul)
- Add topo I incubate 20 min in RT 37°C water bath (mix w/ p20 15ul)
 - Run topo I titration to determine [topo I] necessary
- Terminate w/ 6ul 10x (5.5% SDS + 110mM EDTA) (mix w/ p20 15ul)
- Proteinase K digest 50ug/ml 37° 30 min, 3ul of 5-fold stock (3mg/ml) (mix w/ p20 15ul)
- Heat samples to 37°C 20 min

Ligase Protocol:

- 10 minute protein/DNA incubation 37°C (mix w/ p20 15ul)
- 2ul ligase, 30 min 37°. {8ul 100mM ATP, 5ul ligase, 57ul water} (mix w/ p20 15ul)
- Terminate w/ 6ul 10x (5.5% SDS + 110mM EDTA) (mix w/ p20 15ul)
- Proteinase K digest 50ug/ml 37° 30 min, 3ul of 5-fold stock (3mg/ml) (mix w/ p20 15ul)
- Heat samples to 37° 20 min

Cocktail: (1)

2x Mix	15ul (2x stock)
DNA	Xul (300ng/6 nM in 30ul)
CTD	3ul (10x stock)
Ligase	2ul
H2O	Xul
Total:	30ul

1% TAE Agarose, Chloroquine Gel: (Large Gel Box: 200 mL gel & 1.5L Buffer)

- Let gel cool in ~50°C bath for ~15 min before adding chloroquine
 - Try 0.5ug/ml in TAE 1% agarose gel initially
 - 10mg/ml stock (protect from light)
 - Add 6x sucrose dye. Load 18ul. (See Chloroquine Gel Appendix)
- Run 50V O/N 14-20 hours (no more than 60V)
 - Protect from light, Run pump after ~40 min, check pump tubing every time
- Wash gel in TAE 20 min (remove excess chloroquine)
- Stain with .5ug/ml Ethidium Bromide in H2O 30 min (5 µl/100 ml)
- Destain in H2O 25-40 min

Initial 2x Assay Condition (adapted from fp assay)

<u>ATP Stock:</u>	<u>2x Mix:</u>	<u>[2x]</u>	<u>1ml</u>
0.1M pH 7.5	1M Tris 7.9	20mM	20ul
aliquots @	80% Glycerol	20%	250ul

-80°C	1M MgCl ₂	12mM	12ul
	10mg/ml BSA*	0.2mg/ml	20ul
	2M KCl	80mM	40ul
	1M DTT	2mM	2ul
	H ₂ O		656ul

*BSA may be an issue for certain CTDs

CTD Storage Buffer:

50 mM Tris 7.9

500 mM KCl

33% Glycerol

1 mM EDTA

Stop Buffer: 10ml

2.2 ml 0.5M EDTA

0.55 g SDS

Large Gel Box:

200ml 1% gel

1.5L TAE buffer

***M. tuberculosis* Gyrase Expression and Purification**

Transformation and Expression

- Transform plasmid into Rosetta P-LysS cells (GyrB) / BL21-DE3 RIL cells (GyrA)
- Innoculate 1L cultures with 10mL of overnight 30°C (30°C for GyrA) culture (both 2XYT media)
- Induce w/ 1mM IPTG at approximately OD 0.4 (37°C for GyrA)
- Grow for 3hrs @ 30°C (18°C o/n for GyrA)
- Harvest cells by centrifugation
- Resuspend in Binding Buffer (A) w/ protease inhibitors added.
- Drop freeze cells in liquid nitrogen

Nickel

- Thaw cells on ice
- Sonicate cells (typical for 6L in 50 mL: 4 x 15s at 5.5, wait 2 mins between pulses)
- Load cell lysate onto Nickels column
- Wash extensively with A800 (100-200 mL, until the UV flatlines)
- Wash with A400 (~50 mL)
- Elute with B400; collect peak in a tube or beaker
- Concentrate elution peak to 2 mL or less. Add TEV, the amount will depend on the size of the peak.
- Dialyze overnight against 1 Liter A400 (A800 for GyrB)

Ortho-Nickel

- wash with A400 (A800 for GyrB), collect the flowthrough
- concentrate the flow-through to less than 1 mL

Size Exclusion

- The night before, wash the S300 sizing column with 150 mL water, then equilibrate it with 150 mL sizing buffer.
- Spin the concentrated flowthrough for 10 minutes at 14kRPM, in a microfuge in the cold room
- Load on to sizing column. Collect 1-2 mL fractions.

Nickel Purification Buffers

M. tb GyrA

A800*	A400*	B400 *
20 mM Tris pH 7.9	20 mM Tris pH 7.9	20 mM Tris pH 7.9
30 mM imidazole pH 8.0	30 mM imidazole pH 8.0	30 mM imidazole pH 8.0
800 mM NaCl	400 mM NaCl	400 mM NaCl
10% glycerol	10% glycerol	10% glycerol

*Immediately before use, add 1 ug/mL pepstatin, 1 ug/mL leupeptin, and 1 mM PMSF to warm buffers. Filter through bottle-top filters. Put in frdge to chill while you prep the nickel column

Sizing buffer

50 mM Tris pH 7.9
500 mM KCl
1 mM EDTA
10% glycerol
2mM BME

M. tb GyrB (No A400 buffer is used, wash and dialyze o/n in “A800”)

“A800” *

“B400” *

20mM Tris 7.9

20mM Tris 7.9

500mM NaCl

500mM NaCl

5mM Imidazole pH 8.0

250mM Imidazole pH 8.0

*Immediately before use, add 1 ug/mL pepstatin, 1 ug/mL leupeptin, and 1 mM PMSF to warm buffers. Filter through bottle-top filters. Put in fridge to chill while you prep the nickel column

Sizing buffer

50 mM Tris pH 7.9
500 mM KCl
1 mM EDTA
10% glycerol
2mM BME

Protein Storage

-All proteins are stored in: 30% Glycerol, 50mM Tris pH 7.9, 500mM KCl

-Small, single use aliquots are prepared, flash frozen with liquid nitrogen and stored at -80°C

-This is achieved by spiking the protein sample (post sizing column & concentration) w/ a solution of: 50mM Tris pH 7.9, 500mM KCl, 70% Glycerol.

Malachite Green ATPase Assay

Malachite Green Solution (50 mL)

17 mg Malachite Green	0.0034%
618 mg Ammonium molybdate tetrahydrate	10 mM
4.125 mL HCl 12.1 M stock	1 M
1.7 mL EtOH 100% stock	3.4%
5 μ L Tween 20	0.01%

- Use MQ + sterilized H₂O
- Measure all reagents (minus Malachite Green) in graduate cylinder
- Mix in glass beaker with stirbar for ~15 minutes after adding Malachite Green
- Steriflip mixture
- Store in the dark at room temperature

Assay Setup:

- Run your favorite assay under your favorite conditions. In this case I used my standard gyrase negative supercoiling activity assay conditions and ran my reaction for 30 minutes at 37°C (30 μ L volume).
- Add 200 μ L Malachite Green Solution
- Incubate 5 minutes at room temperature in the dark
- Read 60 μ L of reaction in flat bottom plate at A620.